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(54) Title: POLYPEPTIDES HAVING α- H-α-AMINO ACID AMIDE RACEMASE ACTIVITY AND NUCLEIC ACIDS ENCODING THE SAME

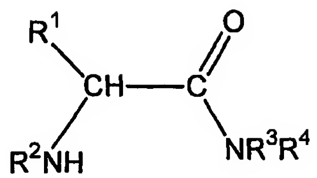
(57) Abstract: The invention relates to isolated polypeptides having α-H-α-amino acid amide racemase activity and nucleic acids encoding the same. The invention also relates to vectors and host cells comprising the nucleic acids according to the invention. The invention also relates to methods of producing and using the polypeptides according to the invention. The invention also relates to a method for isolating polypeptides having α-H-α-amino acid amide rasemase activity, for isolating nucleic acids encoding the same and for isolating microorganisms comprising polypeptides having α-H-α-amino acid acid amide rasemase activity. The invention also relates to new microorganisms comprising polypeptides having α-H-α-amino acid amide racemase activity.

POLYPEPTIDES HAVING α-H-α-AMINO ACID AMIDE RACEMASE ACTIVITY AND NUCLEIC ACIDS ENCODING THE SAME.

The invention relates to isolated polypeptides having α-H-α-amino acid amide racemase activity and nucleic acids encoding the same. The invention also relates to vectors and host cells comprising the nucleic acids according to the invention. The invention also relates to methods of producing and using the polypeptides according to the invention. The invention also relates to a method for isolating polypeptides having α-H-α-amino acid amide racemase activity, for isolating nucleic acids encoding the same and for isolating microorganisms comprising polypeptides having α-H-α-amino acid amide racemase activity. The invention also relates to new microorganisms comprising polypeptides having α-H-α-amino acid amide racemase activity.

α-H-α-Amino acid amides are readily available compounds and are important precursors in the production of pharmaceuticals and of α-H-α-amino acids. For example, enantiomerically enriched α-H-α-amino acids can be obtained from mixtures of D- and L-α-H-α-amino acid amides with randomly chosen enantiomeric excess (ee) by enantioselective enzymatic hydrolysis of one of the enantiomers of the α-H-α-amino acid amide. In such a process for the preparation of enantiomerically enriched α-H-α-amino acids, simultaneous racemization of the α-H-α-amino acid amides would be of great advantage because then complete conversion of the α-H-α-amino acid amide into the desired optically active α-H-α-amino acid is possible. Also, in other processes racemization of α-H-α-amino acid amides is often desired. As enzymatic racemization is preferable over chemical racemization (mild reaction conditions, environmental benefits etc.), many attempts have been made to identify microorganisms with α-H-α-amino acid amide racemase activity and to isolate polypeptides with α-H-α-amino acid amide racemase activity and genes encoding this activity.

For the purpose of the present invention, α-H-α-amino acid amide racemase activity is defined as the ability to catalyze the racemization of an enantiomerically enriched α-H-α-amino acid amide according to formula 1,



(1)

wherein R^1 stands for an optionally substituted alkyl of 1-20 C-atoms or an optionally substituted (hetero)aryl of 3-20 C-atoms (C-atoms of the substituents included) and wherein R^2, R^3 and R^4 each independently stand for H or an optionally substituted alkyl of 1-20 C-atoms or an optionally substituted (hetero)aryl of 3-20 C-atoms (C-atoms of the substituents included) and wherein R^1 may form a ring with R^2 and/or R^3 may form a ring with R^4 together with the carbon and/or nitrogen atoms to which they are bound. Each ring is preferably 5-8 membered. Preferably, R^1, R^2, R^3 or R^4 each independently stand for an alkyl of 1-10 C-atoms or a (hetero)aryl of 3-10 C-atoms (C-atoms of the substituents included). Substituens on the alkyl or (hetero) aryl may be chosen from the group of: hydroxy, alkoxy, mercapto, thioalkyl, alkyl, carboxy, amino, imino, nitro, halogen, carbamoyl, cyano, acyl, peroxy, sulpho or phospho. Examples of α-H-α-amino acid amides according to formula 1 are: proline amide, leucine amide, glutamic acid amide, phenylalanine amide, t-leucine amide, methionine amide, tryptophan amide, leucyl-glycine, valyl-glycine, valyl-alanine, leucyl-alanine.

With α-H-α-amino acid amide racemase is meant a polypeptide having α-H-α-amino acid amide racemase activity.

α-H-α-Amino acid amide racemase activity can be detected with methods similar to methods employed to determine racemizing activity of racemases acting on compounds other than α-H-α-amino acid amides. Such methods are known to the person skilled in the art. For example, by measuring the decrease in enantiomeric excess of an L-α-H-α-amino acid amide or of a D-α-H-α-amino acid amide, α-H-α-amino acid amide racemase activity can be determined.

In Fukumura *et al*, 1977, Agric. Biol. Chem., vol. 41, p 1509-1510, an α-amino-ε-caprolactam racemase isolated from *Achromobacter obae* is disclosed; the nucleic acid sequence coding for this enzyme and the amino acid sequence of this enzyme were published in Naoko *et al.*, 1987, Biochemistry of vitamin B6, p 449-452. For its enzymatic activity, the α-amino-ε-caprolactam racemase isolated from *Achromobacter obae* requires pyridoxal-5-phosphate as a cofactor (Ahmed *et al.*, 1983,

Agric. Biol. Chem., vol 47, p 1887-1893).

The α-amino-ε-caprolactam racemase isolated from *Achromobacter obae* is known to exclusively catalyze the racemization of α-amino-ε-caprolactam. Although the α-amino-ε-caprolactam racemase from *Achromobacter obae* was tested for α-H-α-amino acid amide racemase activity with the following substrates, which are α-H-α-amino acid amides according to formula 1: L-tryptophan amide, L-leucine amide, L-leucyl-glycine, L-valyl-glycine, L-valyl-L-alanine, L-leucyl-L-alanine, no α-H-α-amino acid amide racemase activity was found even when an excess amount of enzyme was used (Ahmed *et al.*, 1983, Agric. Biol. Chem., vol 47, p 1887-1893).

EP-A-383403 discloses α-H-α-amino acid amide racemase activity in the genus *Klebsiella* and related genera and EP-B1-378592 discloses α-H-α-amino acid amide racemase activity in *Arthrobacter sp.* ATCC 31652 (DSM 4639) and in *Corynebacterium sp.* ATCC 31662 (DSM 4640). However, using the microorganisms disclosed in these publications, no racemization could be detected. Therefore, the documents EP-A-383403 and EP-B1-378592 should not be considered as prior art.

So, although much work has been done to find an α-H-α-amino acid amide racemase, up till now, not one α-H-α-amino acid amide racemase has been disclosed.

The invention now provides such α-H-α-amino acid amide racemases.

Applicants surprisingly also discovered a suitable method to isolate micoorganisms displaying α-H-α-amino acid amide racemase activity, in which a microorganism containing sample is enriched by using D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source and in which the thus found cultures are tested for α-H-α-amino acid amide racemase activity.

A suitable way for carrying out such a method is for example as follows; this method will be further referred to as the enrichment method. To find polypeptides according to the invention a microorganism containing sample, e.g. an environmental sample, for example a soil sample or a waste water sample is cultured in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source until growth can be detected.

The sample can be directly added onto/into the suitable growth

medium with D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source. There are also other ways of applying the sample to the medium, for example by adding the filtrate of a wash solution used to wash the sample with.

The culturing of the environmental sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as the sole nitrogen source is a so-called enrichment and was described by Fukumura *et al.*, 1977, Agric. Biol. Chem., vol 41, p 1321-1325, who used this enrichment in a method to isolate α-amino-ε-caprolactam racemases. Preferably, the enrichment is continued by one or more transfers of the cultured microorganism(s) into or onto a 'fresh' suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as the sole nitrogen source until a monoculture (as opposed to mixculture) is reached. Typically, this will be after 4 or 5 transfers.

It may be of advantage to add other nitrogen sources than D- or a mixture of D- and L-α-amino-ε-caprolactam, for example to help the cultures to start growing. For a good enrichment, however, the D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam is present in such amounts that microorganisms having the ability to convert D- and/or L-α-amino-ε-caprolactam, can continue to grow, whereas other microorganisms do not.

The person skilled in the art knows how to choose the culture conditions, for instance the conditions of the culture may depend on the source of the environmental sample and/or on the desired conditions of the desired process.

The microorganisms obtained by enrichment are tested for α-H-α-amino acid amide racemase activity. This testing for α-H-α-amino acid amide racemase activity can be done directly on the whole cells or on permeabilized cells of the colonies obtained after plating the cultured microorganisms. Alternatively, the colonies obtained are cultured separately in a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source, after which cell free extract is prepared therefrom, which is subsequently tested for α-H-α-amino acid amide racemase activity.

Cell free extract can be prepared according to standard methods known to the person skilled in the art, e.g. by sonification, French press etc. Cell permeabilization can be obtained according to standard methods known to the person

skilled in the art, e.g. by addition of small amounts of toluene. Suitable growth media are in fact all media, which contain as a or as the sole nitrogen source D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam. Suitable growth media are well known in the art. They can for example be composed by the person skilled in the art with guidance from Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual.2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Testing for α-H-α-amino acid amide racemase activity is preferably performed on a monoculture, but can also be performed on a mixculture.

In a preferred embodiment of the invention, the mix and/or monocultures obtained after the enrichment method, are tested for the ability to use L-α-amino-ε -caprolactam and for the ability to use D-α-amino-ε-caprolactam as a or as the sole nitrogen source.

Therefore, the invention also relates to a method for isolating microorganisms displaying α-H-α-amino acid amide racemase activity, comprising the steps of:

a) Culturing, in one or more transfer steps, a microorganisms containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source.
b) Testing the thus obtained microorganisms for α-H-α-amino acid amide racemase activity.

Surprisingly, it was found that the enrichment method is suitable for the isolation of microorganisms displaying α-H-α-amino acid amide racemase activity. This is the more surprising since this enrichment selects for α-amino-ε-caprolactam racemase activity and not for α-H-α-amino acid amide racemase activity. Therefore, the invention also relates to new microorganisms (comprising polypeptides) having α-H-α-amino acid amide racemase activity obtainable with said method.

Several microorganisms might be isolated this way, for example it may be possible to isolate microorganisms from the following genera: *Agrobacterium, Ochrobactrum, Arthrobacter, Micrococcus, Aureobacterium, Corynebacterium, Rhodococcus, Brevibacterium, Rubrobacter, Nocardioides, Terrabacter.*

Several monocultures of strains of microorganisms displaying α-H-α-amino acid amide racemase activity were isolated with the enrichment method and these microorganisms were deposited under the Budapest Treaty with The National

Collections of Industrial and Marine Bateria Limited (NCIMB), Aberdeen, Scotland, on May 8th, 2002: *Agrobacterium rhizogenes* Na was deposited under number NCIMB 41127, *Agrobacterium rhizogenes* Bi was deposited under number NCIMB 41128, *Arthrobacter nicotianae* was deposited under number NCIMB 41126, *Ochrobactrum anthropi* 1A was deposited under number NCIMB 41129.

From isolated microorganisms obtained with the enrichment method it is possible to isolate a nucleic acid sequence encoding a polypeptide having α-H-α-amino acid amide racemase activity and to clone and express this nucleic acid to produce a polypeptide according to the invention. The isolation of a nucleic acid sequence and subsequent recloning and expression thereof can be done according to standard methods, which are known to the person skilled in the art.

Therefore, the invention also relates to a method for isolating a nucleic acid sequence encoding a polypeptide with α-H-α-amino acid amide racemase activity, comprising the steps of:

a) Culturing in one or more transfer steps, a microorganism containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source.

b) Testing the thus obtained microorganism(s) for α-H-α-amino acid amide racemase activity.

c) Isolating a nucleic acid sequence encoding an α-H-α-amino acid amide racemase from the obtained microorganism(s) in a manner known per se.

Isolating a nucleic acid sequence encoding a polypeptide having α-H-α-amino acid amide racemase activity from a microorganism having α-H-α-amino acid amide racemase activity can for example be done by preparing a DNA library, a cDNA library or an expression library from the microorganism(s) having α-H-α-amino acid amide racemase activity, by (partial) purification of the polypeptide having α-H-α-amino acid amide racemase activity followed by reversed genetics or by using nucleic acid arrays. These techniques are all known to the person skilled in the art. For example, after the preparation of a DNA or cDNA library, the nucleic acid encoding a polypeptide having α-H-α-amino acid amide racemase activity may be selected by using a probe or a PCR-primer based on sequence information of a homologous gene or on sequence information of a polypeptide having α-H-α-amino acid amide racemase activity. For example, after the preparation of an expression library, the clone of the library having α-H-α-amino acid amide racemase activity may be selected by using a α-H-α-amino

acid amide racemase activity assay, by using the enrichment method of the invention or by using antibodies raised against a polypeptide having α-H-α-amino acid amide racemase activity. For example, reversed genetics can be performed by the purification and sequencing of (part of) the polypeptide having α-H-α-amino acid amide racemase activity and isolating the desired nucleic acid sequence based on the sequence information of the polypeptide having α-H-α-amino acid amide racemase activity, for example with a probe or a PCR-primer. For example, DNA arrays may be used to derive the nucleic acid sequence encoding a polypeptide having α-H-α-amino acid amide racemase activity, if the genomic sequence of the microorganism having α-H-α-amino acid amide racemase activity is known by comparing the expression pattern of the microorganism under conditions under which the microorganism does not display α-H-α-amino acid amide racemase activity to conditions under which the microorganism does display α-H-α-amino acid amide racemase activity.

In one embodiment of the invention, the nucleic acid sequence encoding an α-H-α-amino acid amide racemase is isolated as follows.

In a first step total DNA is isolated and a gene library is prepared from the microorganisms obtained by the enrichment method and expressed in a suitable vector in a suitable host (e.g. as described in the examples). In a second step, the clones of the gene library containing a vector with insert (the insert is a piece of DNA isolated from the microorganisms) are tested on the ability to catalyse the racemization of an α-H-α-amino acid amide. For example, testing of the clones for α-H-α-amino acid amide racemase activity can be done according to the methods as described in the examples.

In subsequent steps, the nucleic acid sequence of the insert of the vector in a clone having the ability to catalyse the racemization of an α-H-α-amino acid amide is determined and open reading frames are identified from the thus determined nucleic acid sequence, after which the open reading frames are recloned and expressed in a suitable vector and in a suitable host and again tested for α-H-α-amino acid amide racemase activity. By expression of the open reading frame encoding α-H-α-amino acid amide racemase activity in a suitable vector in a suitable host a polypeptide having α-H-α-amino acid amide racemase activity according to the invention can be produced.

With the enrichment method and subsequent isolation of the nucleic acid sequence, the nucleic acid sequence encoding an α-H-α-amino acid amide

racemase from a strain identified as *Ochrobactrum anthropi* 1A deposited under number NCIMB 41129 with the NCIMB was obtained. This nucleic acid sequence is presented in SEQ ID: NO. 1. The amino acid sequence of the corresponding polypeptide is presented in SEQ ID: NO. 2. It was found that the α-H α-amino acid amide racemase, with its amino acid sequence presented in SEQ ID: NO. 2, is active over a broad pH and temperature range. With this enrichment method, also the nucleic acid sequence encoding an α-H-α-amino acid amide racemase from a strain identified as *Arthrobacter nicotianae* deposited under number NCIMB 41126 with the NCIMB was identified (SEQ ID: NO. 8). The amino acid sequence of the corresponding polypeptide is presented in SEQ ID: NO. 9.

The invention also relates to nucleic acid sequences encoding polypeptides with α-H α-amino acid amide racemase activity obtainable by the above method.

A nucleic acid sequence of encoding a polypeptide with α-H-α-amino acid amide racemase activity according to the invention, such as a nucleic acid sequence with the sequence of SEQ ID: NO. 1 or of SEQ ID: NO. 8 may also be isolated using standard molecular biology techniques and the sequence information provided herein. For example, using all or a portion of the nucleic acid sequence of SEQ ID: NO. 1 or SEQ ID: NO. 8 as a hybridization probe, a nucleic acid sequence according to the invention can be isolated using standard hybridization and cloning techniques (e. g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual.2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid sequence encompassing all or a portion of SEQ ID: NO. 1 or SEQ ID: NO. 8 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence information contained in SEQ ID: NO. 1 or SEQ ID: NO. 2, respectively SEQ ID: NO. 8 or SEQ ID: NO. 9.

A nucleic acid sequence of the invention can be amplified using for example genomic DNA, cDNA or alternatively mRNA as a template and appropriate oligonucleotide primers according to standard (RT)-PCR amplification techniques. The nucleic acid so amplified can be cloned into a suitable vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to or hybridizable to

nucleic acid sequences according to the invention can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

It should be noted that the invention also includes mutants of the nucleic acids encoding polypeptides having α-H-α-amino acid amide racemase activity, which have one or more mutations as compared to the corresponding polypeptide isolated from a naturally occurring host. Methods for making mutations are known to the person skilled in the art, e.g. by random mutagenesis (for example by error prone PCR or by UV radiation), site directed mutagenesis, etc.

The invention also relates to a method for producing a polypeptide with α-H-α-amino acid amide racemase activity, comprising the steps of:

a) Culturing in one or more transfer steps, a microorganism containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source.
b) Testing the thus obtained microorganisms for α-H-α-amino acid amide racemase activity.
c) Isolating a nucleic acid sequence encoding an α-H-α-amino acid amide racemase fron the obtained microorganism(s) in a manner known per se.
d) Expressing the nucleic acid sequence in a suitable host to produce a polypeptide with α-H-α-amino acid amide racemase activity.

Expressing the nucleic acid sequence encoding a polypeptide according to the invention, can for example be done by cloning the nucleic acid in a suitable vector and after introduction in a suitable host, (over)expressing the sequence according to standard cloning and expression techniques, which are known to the person skilled in the art (e.g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual.2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), to produce a polypeptide according to the invention. Therefore, the invention also relates to vectors comprising a nucleic acid sequence according to the invention.

Alternatively, to produce a polypeptide according to the invention, the nucleic acid sequence encoding a polypeptide according to the invention can be integrated into the genome of a host cell and be (over)expressed. This can be done according to methods known to the person skilled in the art. Therefore, the invention also relates to a host cell comprising and expressing a nucleic acid sequence according to the invention, preferably to a host cell comprising a vector comprising a

nucleic acid sequence according to the invention.

Alternatively, a polypeptide according to the invention can be overexpressed in its natural host, for example by placing a suitable promoter upstream of the nucleic acid sequence according to the invention, by integrating one or more copies of a nucleic acid sequence according to the invention into the genome of its natural host, preferably by expressing a nucleic acid according to the invention in a suitable vector in its natural host, which are all methods known to the person skilled in the art.

Suitable hosts are the hosts normally used for cloning and expression and are known to the person skilled in the art. Examples of suitable *E. coli* host strains are: TOP10F', TOP10, DH10B, DH5α, HB101, W3110, BL21(DE3) and BL21(DE3)pLysS.

Suitable vectors are the vectors normally used for cloning and expression and are known to the person skilled in the art. Examples of suitable vectors for expression in *E. coli* are given e.g. in table 1 in Makrides, S.C., 1996, Microbiological Reviews, p. 512-538. Preferably, the vector contains a promoter upstream of the cloning site containing the nucleic acid sequence encoding the polypeptide with α-H-α-amino acid amide racemase activity, which can be switched on after the host has been grown to express the corresponding polypeptide having α-H-α-amino acid amide racemase activity. Promoters, which can be switched on and off are known to the person skilled in the art and are for example the *lac* promoter, the *ara*BAD promoter, the T7 promoter, the *trc* promoter, the *tac* promoter, the *trp* promoter, and the *aro*H promoter.

The invention therefore relates to polypeptides with α-H-α-amino acid amide racemase activity obtainable by the above method.

In a preferred embodiment of the invention, the invention relates to isolated polypeptides having α-H-α-amino acid amide racemase activity and having a degree of identity with the amino acid sequence presented in SEQ ID: NO. 2 of at least about 35%, preferably of at least about 40%, more preferably of at least about 50%, even more preferably of at least about 55%, in particular of at least about 65%, more in particular of at least about 75%, even more in particular of at least about 85%, even more in particular of at least about 90%, even more in particular of at least about 95%, most in particular of at least about 97%.

In another preferred embodiment of the invention, the invention

relates to isolated polypeptides having α-H-α-amino acid amide racemase activity and having a degree of identity with the amino acid sequence presented in SEQ ID: NO. 9 of at least about 50%, preferably of at least about 60%, more preferably of at least about 70%, even more preferably of at least about 80%, in particular of at least about 90%, more in particular of at least about 95%, most in particular of at least about 97%.

For purpose of the present invention, the degree of identity between two amino acid sequences is determined by the blastp pairwise alignment algorithm (NCBI) with an identity table and the following alignment parameters: mismatch = -3 penalty = -3, gap extend = 1, match bonus = 1, Gap x – droff = 50, expect = 10, wordsize = 3.

The present invention also relates to isolated polypeptides having α-H-α-amino acid amide racemase activity, which are encoded by nucleic acid sequences which hybridize under low stringency conditions, preferably under medium stringency conditions, more preferably under high stringency conditions and most preferably under very high stringency conditions with the coding sequence of SEQ ID: NO. 1 or a complementary strand thereof or with the coding sequence of SEQ ID: NO. 8 or a complementary strand thereof.

Hybridization experiments can be performed by a variety of methods, which are well available to the skilled man. General guidelines for choosing among these various methods can be found in e.g. chapter 9 of Sambrook, J., Fritsh, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual.2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

With stringency of the hybridization conditions is meant, the conditions under which the hybridization, consisting of the actual hybridization and wash steps, are performed. Wash steps are used to wash off the nucleic acids, which do not hybridize with the target nucleic acid immobilized on for example a nitrocellulose filter. The stringency of the hybridization conditions can for example be changed by changing the salt concentration of the wash solution and/or by changing the temperature under which the wash step is performed (wash temperature). Stringency of the hybridization increases by lowering the salt concentration in the wash solution or by raising the wash temperature. For purpose of this application, the hybridization is performed in 6 X sodium chloride/sodium citrate (SSC) at about 45°C for about 12 hours. Two consecutive 30 minutes wash steps in 1 X SSC, 0.1% SDS at 50°C is an example of low stringency, at 55°C an example of medium stringency, at 60°C an

example of high stringency, at 65°C an example of very high stringency.

The present invention also relates to isolated polypeptides having α-H-α-amino acid amide racemase activity and which display immunological cross-reactivity with an antibody raised against a fragment of the amino acid sequence according to SEQ ID: NO. 2 or SEQ ID: NO. 9.

The immunological cross reactivity may be assayed using an antibody raised against, or reactive with, at least one epitope of the isolated polypeptide according to the present invention having α-H-α-amino acid amide racemase activity. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson *et al.*, Practical Immunology, Third Edition (1989), Blackwell Scientific Publications. The immunochemical cross-reactivity may be determined using assays known in the art, an example of which is Western blotting, e.g. as described in Hudson *et al.*, Practical Immunology, Third Edition (1989), Blackwell Scientific Publications.

The invention also relates to fragments of the polypeptides according to the invention and having α-H-α-amino acid amide racemase activity of at least 100 amino acids, preferably of 125 to 350 amino acids, more preferably of 200 to 300 amino acids.

The invention also relates to fusion proteins made by expression of a nucleic acid sequence encoding a polypeptide according to the invention operatively linked to one or more nucleic acid sequences, which encode (a) marker polypeptide(s). With operatively linked is meant, that the nucleic acid sequences are linked such that, if expressed, the polypeptide according to the invention with the marker polypeptide(s) on its N- and/or C-terminus is produced. The marker polypeptide can serve many purposes, for example, it may be used to increase the stability or the solubility of the fusion protein, it may be used as a secretion signal, which is a signal that directs the fusion protein to a certain compartment in the cell or it may be used to facilitate purification of the fusion protein. An example of a marker polypeptide used to facilitate purification of the fusion protein is the hexahistidine peptide. The purification of a fusion protein with a hexahistidine tag is for example described in Gentz *et al.*, 1989, Proc. Natl. Acad. Sci. USA, vol. 86, p. 821-824. A fusion protein with a hexahistidine tag can for example be produced in a pQE vector (Qiagen, Inc.), by following the protocol of the supplier.

The invention also relates to nucleic acid sequences encoding

polypeptides having α-H-α-amino acid amide racemase activity according to the invention. These nucleic acid sequences may be obtained with the enrichment method and subsequent isolation of the desired nucleic acid as described above.

α-H-α-amino acid amide racemases and microorganisms displaying α-H-α-amino acid amide racemase activity according to the invention can be used in a process for the racemization of an enantiomerically enriched α-H-α-amino acid amide. Therefore, the invention relates to a process for the racemization of an enantiomerically enriched α-H-α-amino acid amide, wherein the racemization is performed in the presence of a polypeptide according to the invention or a microorganism according to the invention.

α-H-α-Amino acid amide racemases according to the invention can suitably be used together with an enantioselective amidase in a process for the preparation of enantiomerically enriched α-H-α-amino acids from a mixture of D- and L-α-H-α-amino acids amides or in a process for the preparation of L-α-H-α-amino acids from the corresponding D-α-H-α-amino acid amides or in a process for the preparation of D-α-H-α-amino acids from the corresponding L-α-H-α-amino acid amides. Use of α-H-α-amino acid amide racemases in such processes will (theoretically) lead to a 100% conversion of the α-H-α-amino acid amide into the corresponding enantiomerically enriched α-H-α-amino acid (100% yield). The invention therefore also relates to the use of an α-H-α-amino acid amide racemase according to the invention in combination with an enantioselective amidase in a process for the preparation of enantiomerically enriched α-H-α-amino acids from a mixture of D- and L-α-H-α-amino acids amides or in a process for the preparation of L-α-H-α-amino acids from the corresponding D-α-H-α-amino acid amides or in a process for the preparation of D-α-H-α-amino acids from the corresponding L-α-H-α-amino acid amides. The invention also relates to a process for the preparation of an enantiomerically enriched α-H-α-amino acid from a mixture of the corresponding D- and L-α-H-α-amino acid amides or the preparation of an L-α-H-α-amino acid from the corresponding D-α-H-α-amino acid amide or the preparation of a D-α-H-α-amino acid from the corresponding L-α-H-α-amino acid amide, wherein the process is performed in the presence of an enantioselective amidase and a polypeptide with α-H-α-amino acid amide racemase activity according to the invention or a microorganisms displaying α-H-α-amino acid amide racemase activity according to the invention.

Preferably, in the above-mentioned processes, the enantioselective amidase is at least 90% enantioselective; more preferably at least 95% enantioselective, even more preferably at least 98% enantioselective, most preferably at least 99% enantioselective. 90% Enantioselectivity of the amidase (for example a D-amidase) is defined for the present invention as the ability of the amidase to convert a racemic mixture of DL-α-H-α-amino acid amides into 90% of one enantiomer of the α-amino acid (e.g. D-α-H-α-amino acid) and into 10% of the other enantiomer of the α-H-α-amino acid (e.g. L-α-H-α-amino acid) at 50% total conversion of the α-H-α-amino acid amide mixture. 95% Enantioselectivity (e.g. 95% D- and 5% L-α-H-α-amino acid) corresponds to an E-value of 58.4 (e.e. 90%). 98% Enantioselectivity (e.g. 98% D- and 2% L-α-H-α-amino acid) corresponds to an E-value of 193.6 (e.e. 96%). 99% Enantioselectivity (e.g. 99% D- and 1% L-α-H-α-amino acid) corresponds to an E-value of 458.2 (e.e. 98%).

For α-H-α-amino acid amide racemase activity, it may sometimes be of advantage to add a cofactor, for example pyridoxal-5-phosphate.

Preferably, in said processes, the α-H-α-amino acid amide racemase according to the invention does not display α-H-α-amino acid racemase activity, i.e. the ability to catalyse the racemization of a D-α-H-α-amino acid and an L-α-H-α-amino acid.

The invention is illustrated by way of the following examples. However, these examples are not meant to restrict the invention.

Examples

General procedures

Standard molecular cloning techniques such as plasmid DNA isolation, gel electrophoresis, enzymatic restriction modification of nucleic acids, *E. coli* transformation etc. were performed as described by Sambrook et al., 1989, "Molecular Cloning: a laboratory manual", Cold spring Harbor Laboratories, Cold Spring Harbor, NY or the supplier's manual. Standard molecular cloning enzymes such as restriction endonucleases, T4 DNA ligase, etc. were obtained from Invitrogen (Breda, The Netherless) unless otherwise stated. Synthetic oligodeoxynucleotides were obtained from Sigma-Genosys (Cambridge, UK), and Invitrogen (Paisley, Scotland, UK). DNA sequence analyses were performed by BaseClear (Leiden, The Netherlands) using the chain termination method with dye-labeled dideoxy-terminators.

Example 1

Enrichment method

In this enrichment procedure, different soil and sludge samples that had been stored at -80°C, were used for direct inoculation of 50 ml of liquid Medium A (Yeast Carbon Base (Difco, 11.7 g/l), KH_2PO_4 (6.7 g/l), K_2HPO_4 (8.9 g/l), pH 7.0), containing racemic DL-α-amino-ε-caprolactam (2 g/l) as sole nitrogen source. Per flask, one spatula of inoculum was used. The different flasks were incubated at 28°C and 200 rpm.

After about 2 days good growth was observed in all flasks. After sedimentation of the soil particles in all cultures, 1:1,500 dilutions were prepared in Medium A containing 2 g/l DL-α-amino-ε-caprolactam, and were incubated at 28°C and 200 rpm. After obtaining sufficient growth, samples were taken from each flask to determine the concentration of both L- and D-α-amino-ε-caprolactam in the culture broths. These samples were centrifuged and the supernatants were filtered through a 0.45 μM filter to remove all cells. Then these samples were analyzed by HPLC. *o*-Phthaldehyde in combination with D-3-mercapto-2-methylpropionic acid was used in this HPLC method as a chiral reagent for the separation of both α-amino-ε-caprolactam enantiomers (Duchateau *et al.*, 1992, J. Chromatogr., vol. 623, p 237 – 245).

Only flasks in which cultures had grown that used both enantiomers

of α-amino-ε-caprolactam were selected for the next steps. Different dilutions of these cultures were plated onto plates with Medium A containing 2 g/l DL-α-amino-ε-caprolactam as sole nitrogen source, and were incubated at 28°C. Colonies were randomly chosen and reisolated on the same type of selective plates to obtain monocultures. Then, a colony of each monoculture was transferred into 5 ml of Medium A containing 2 g/l DL-α-amino-ε-caprolactam. After 3 days of incubation at 28°C and 200 rpm, the cultures obtained were analyzed for use of D- and L-α-amino-ε-caprolactam exactly as described above. The monocultures that could use both D- and L-α-amino-ε-caprolactam were stored at -80°C in 20% (v/v) glycerol.

Cells from these monocultures were finally tested for α-amino-ε-caprolactam racemase activity. After cultivation in Medium A containing DL-α-amino-ε-caprolactam (2 g/l) at 28°C and 200 rpm for three days, cells were harvested by centrifugation, washed with 20 mM HEPES-NaOH buffer (pH 7.7) and lyophilized.

Then 100 mg of these lyophilized cells from each monoculture were added to a reaction mixture consisting of 10 mM HEPES-NaOH buffer (pH 7.7), toluene (2,5 v/v %), pyridoxal-5-phosphate (20 μM), and L-α-amino-ε-caprolactam (2.5 m%). Chemical blanks (with the assay mixture without the lyophilized cells) were used as a negative control. All reaction mixtures (including the blanks) were incubated for approximately 72 hours at 40°C before the reaction was stopped by the addition of 9 volumes of 1 M H_3PO_4. After removal of the cells by filtration through a 0.45 μM filter, these reaction mixtures were analyzed by the HPLC method described above to determine the concentration of D- and L-α-amino-ε-caprolactam. Finally, monocultures that formed D-α-amino-ε-caprolactam from the L-α-amino-ε-caprolactam substrate were sent to the NCIMB for strain determination.

Nb. no D-α-amino-ε-caprolactam could be determined in the chemical blanks.

Strain determination

One of the thus obtained monocultures that could racemize L-α-amino-ε-caprolactam was identified as an *Ochrobactrum anthropi* strain by the NCIMB and was deposited under the Budapest Treaty as *Ochrobactrum anthropi* IA with the NCIMB under number NCIMB 41129. The results of the determination are presented in table 1.

Table 1: Results of strain identification of isolate IA by National Collections of Industrial and Marine Bacteria Limited (NCIMB LTD). Identification number ID4036.

Isolate code	IA
Incubation temperature (°C)	30
Gram stain	-
Spores	-
Motility	+
Colony morphology (after 2 days growth on LB medium)	Round, regular, entire, smooth, shiny, low convex, buff, opaque. 1 mm diameter.
Growth at 37°C	+
Growth at 45°C	-
Catalase	+
Oxidase	+
Fermentative in glucose OF	Oxidative
Results from API 20NE test:	
NO_3 reduction	+
Indole reduction	-
Acid from glucose	-
Arginine dihydroxylase	-
Urease	+
Aesculin hydrolysis	-
Gelatin hydrolysis	-
β-galactosidase	-
Glucose assimilation	+
Arabinose assimilation	+
Mannose assimilation	+
Mannitol assimilation	+
N-acetyl-glucosamine assimilation	+
Maltose assimilation	-
Gluconate assimilation	-
Caprate assimilation	-
Adipate assimilation	-
Malate assimilation	+

Isolate code	IA
Citrate assimilation	+
Phenylacetate assimilation	-
Cytochrome oxidase	+
Results from further biochemical tests:	
Acid production in:	
Glucose ASS[a]	+
Dulcitol ASS	+
Adonitol ASS	+
Raffinose ASS	-
Xylose OF	+
Glycine CSU[b]	+
Nitrite reduction to N_2	+
PPA	+
Simmons citrate	-
Tween 80 hydrolysis	-
Tyrosine decomposition	-
H_2S production (PbAc)	+

[a]: ASS, ammonia salt sugar.

[b]: CSU, carbon source utilization.

Measurement of α-H-α-amino acid amide racemase activity of lyophilized *Ochrobactrum anthropi* IA cells

O. anthropi IA cells were cultivated in Medium A containing DL-α-amino-ε-caprolactam (2 g/l) as sole nitrogen source. After incubation at 28°C and 200 rpm for three days ($OD_{620\,nm}$ = 4.7), cells were harvested by centrifugation, washed in 20 mM HEPES-NaOH buffer of pH 7.7 and lyophilized.

The lyophilized cells were used to demonstrate α-H-α-amino acid amide racemase activity. The assay mixture consisted of 10 mM HEPES-NaOH buffer (pH 7.7) containing toluene (2,5 v/v %), pyridoxal-5-phosphate (20 μM), D- or L-leucine amide (2.5 m%) and in some cases EDTA (20 mM). Per assay 150 mg of lyophilized cells were used. Chemical blanks (with the assay mixture without the *O. anthropi* IA cells) were used as a negative control. All reaction mixtures (including the blanks) were incubated for approximately 72 hours at 40°C before the reaction was stopped by the

addition of 9 volumes of 1 M H_3PO_4. Finally, the reaction mixtures were analyzed by HPLC to determine the concentrations of D- and L-leucine amide and of D- and L-leucine. *o*-Phthaldehyde in combination with D-3-mercapto-2-methylpropionic acid was used in this HPLC method as a chiral reagent for the enantioseparation of these amino compounds (Duchateau *et al.*, 1992, J. Chromatogr., vol. 623, p 237 – 245).

In a number of reactions EDTA was added to inhibit L-and/or D-specific amidase(s) present in the *O. anthropi* IA cells. By inhibiting this amidase, the L- and/or D-amino acid amide is not or just partly converted into the corresponding amino acid thereby enabling the demonstration of amino acid amide racemase activity by the detection of the other amino acid amide enantiomer. These reactions with EDTA (No. 3 & 4), clearly showed that *O. anthropi* IA cells contain an α-H-α-amino acid amide racemase activity, because D-leucine amide was converted into L-leucine amide and vice versa. The chemical blanks did not show this racemization reaction.

Without EDTA, amidases convert the substrate and/or product α-H-α-amino acid amides into α-H-α-amino acids thereby preventing direct detection of the other α-H-α-amino acid amide enantiomer as racemization product. Starting from D-leucine amide, however, the reaction without EDTA also clearly proved the presence of an α-H-α-amino acid amide racemase activity in *O. anthropi* IA, because this substrate was converted to significant amounts of L-leucine, and a control experiment excluded its formation via D-leucine, because *O. anthropi* IA did not racemize leucine. Formation of L-leucine from D-leucine amide could not be detected in the chemical blank.

Table 2: α-H-α-amino acid amide racemase activity in lyophilized *O. anthropi* IA cells.

No.	Substrate	EDTA (20 mM)	D-leucine amide (m%)	L-leucine amide (m%)	D-leucine (m%)	L-leucine (m%)
1	D-leucine amide	-	0.22	<0.001	0.015	0.044
2	L-leucine amide	-	<0.001	<0.001	0.001	0.21
3	D-leucine amide	+	0.24	0.032	0.007	0.008
4	L-leucine amide	+	0.021	0.15	0.003	0.058

The concentrations given are the concentrations measured in the reaction mixtures, which are 10 times diluted as compared to the initial reaction mixtures.

Example 2

Enrichment method

Approximately 30 g of fresh soil samples from different locations were resuspended in 50 ml of Medium A, supplemented with 2 g/l D-α-amino-ε-caprolactam as sole nitrogen source, and shaken for 30 minutes at 4°C and 200 rpm. The suspensions were filtered through a filter paper to remove the soil particles. With the filtrates obtained the following two different approaches were used to select for strains that could use D-α-amino-ε-caprolactam as nitrogen source:

- 5 ml Of the different filtrates were transferred to empty 100 ml flasks. All flasks were incubated at 28°C and 200 rpm until an $OD_{600\ nm}$ was reached of approximately 2.5. Then the cultures were diluted 1:100 in fresh Medium A containing 2 g/l D-α-amino-ε-caprolactam, again followed by a cultivation step. These dilution and recultivation step was repeated 3 more times. Then different dilutions of good growing cultures were plated onto plates with Medium A containing 2 g/l D-α-amino-ε-caprolactam as sole nitrogen source, that were incubated at 28°C. Colonies were randomly chosen and reisolated 5 times onto identical selective plates, yielding pure monocultures. Finally, these monocultures were stored at –80°C in 20% (v/v) glycerol.
- As alternative enrichment strategy, different dilutions of all filtrates were plated directly onto Medium A containing 2 g/l D-α-amino-ε-caprolactam as sole nitrogen source. After 2 days of incubation at 28°C, first colonies appeared on almost all plates. After a few more days to 3 weeks, a lot of morphologically different colonies were obtained. Morphologically different colonies were randomly chosen and reisolated 5 times onto identical selective plates, yielding pure monocultures. Finally, these monocultures were stored at –80°C in 20% (v/v) glycerol.

Cells from these monocultures were finally tested for α-amino-ε-caprolactam racemase activity. After cultivation in 800 ml of Medium A containing 2 g/l of D-α-amino-ε-caprolactam at 28°C and 200 rpm, cells were harvested by centrifugation (20 min. at 5,000 x g, 4°C), and resuspended in 40 ml of sonification buffer (NaCl, 16 g/l; KCl, 0.74 g/l; Na_2HPO_4, 0.27 g/l; glucose, 2 g/l; HEPES, 10 g/l, pH 7.0). Then the cells were disintegrated by sonification using a Soniprep 150 of MSE at

an amplitude of 20 microns in cycles of 10 min. using time intervals of 10 sec. (i.e. 10 sec. sonification, 10 sec. cooling) and cooling in ice/acetone. After each cycle, the cells were viewed microscopically and the sonification procedure was stopped when 50 - 70% of the cells were broken.

Then D-α-amino-ε-caprolactam and pyridoxal-5-phosphate were added to the disintegrated cell suspensions of each monoculture to concentrations of 5 g/l and 0.01 mM respectively. After 1.5 h of incubation at 20°C and 20 rpm, 2 ml samples were transferred into 1 ml of 1 M H_3PO_4. After removal of all particulates by centrifugation followed by filtration through a 0.22 μM filter, concentrations D- and L-α-amino-ε-caprolactam in the samples were determined by HPLC using the protocol as described in example 1. Finally, monocultures that formed L-α-amino-ε-caprolactam from the D-α-amino-ε-caprolactam substrate were sent to the NCIMB for strain determination.

Strain determination

The thus obtained monocultures were identified by the NCIMB as *Agrobacterium rhizogenes* (*Agrobacterium rhizogenes* Bi and *Agrobacterium rhizogenes* Na and *Arthrobacter nicotianae* via 16S rDNA sequence determination. The results of this 16S rDNA determination are presented below. The microorganisms were deposited under the Budapest Treaty with the NCIMB. *Agrobacterium rhizogenes* Na was deposited under number NCIMB 41127, *Agrobacterium rhizogenes* Bi under number NCIMB 41128 and *Arthrobacter nicotianae* under number NCIMB 41126.

Analysis of 16S rDNA sequence

Methods

DNA extraction: The DNA was extracted using the Prepman purification kit and stored on ice until use.

Polymerase chain reaction: The 16S ribosomal DNA gene was amplified using universal eubacterial primers and analysed by electrophoresis on a 1% agarose gel.

DNA clean-up: The 500 bp fragment was purified by spin column centrifugation and resuspended in sterile destilled water.

DNA sequencing: The purified DNA product was automatically sequenced using the dideoxy chain terminator method (Sanger *et al.*, 1997).

Sequence analysis by database comparison: The sequence of the 16S rDNA gene was

compared with nucleic acid sequence databases.

Results

The 16S rDNA sequences of *Agrobacterium rhizogenes* Na NCIMB 41127 (SEQ ID: NO. 5), *Agrobacterium rhizogenes* Bi NCIMB 41128 (SEQ ID: NO. 4), and *Arthrobacter nicotianae* NCIMB 41126 (SEQ ID: NO. 3) are presented in the sequence listing part.

Measurement of α-H-α-amino acid amide racemase activity of disintegrated cells of *Agrobacterium rhizogenes* Na, *Agrobacterium rhizogenes* Bi, *Arthrobacter nicotianae*

Preparation of disintegrated cells

After growth of these three strains in Medium A containing 2 g/l of D-α-amino-ε-caprolactam, cells were harvested by centrifugation at 12.000 x g. After centrifugation the supernatant was decanted and the wet weight of the pellet was measured. To the pellet, sonification buffer (NaCl, 16 g/l; KCl, 0.74 g/l; Na_2HPO_4, 0.27 g/l; glucose, 2 g/l; HEPES, 10 g/l, pH 7.0) was added in a 1:2 ratio (wet weight cell pellet : ml buffer). During storage between cell harvest and sonification the pellet was frozen in the sonification buffer at –86°C.

Sonification was performed with a Soniprep 150 of MSE at maximal amplitude of 20 microns in periods of 10 min. using time intervals of 10 sec. (i.e. 10 sec. sonification, 10 sec. cooling down), cooling the sample on ice/acetone. Each 10 min. the cells were viewed microscopically. The sonification procedure was stopped when >70% of the cells were broken.

α-H-α-Amino acid amide racemase activity test with disintegrated cells

1 ml Of disintegrated cells of each of the three strains was incubated in a HEPES-NaOH buffer (20 mM) pH 7.7, to which pyridoxal-5-phosphate (0.01 mM) was added as a cofactor and D-leucine amide (60.8 mM) as a substrate in a total volume of 10 ml (0.79 w/w% D-leucine amide). After 0 and 139 hours 1 ml samples were taken (of which the exact weight was measured) and the reaction in these samples was stopped by adding 1 ml methanol (of which the exact weight was measured too; to be able to calculate dilution factors). Stopped reaction mixtures were

centrifuged to remove particulates. The supernatants were frozen at -86°C until HPLC analysis according to the method given in example 1.

Blank reaction mixtures were prepared, sampled and stopped in the same way, with the exception that no substrate was added and that instead of disintegrated cells, only HEPES-NaOH buffer (20 mM, pH 7.7) was added.

The results are shown in table 3.

Table 3. Measurement of α-H-α-amino acid amide racemase activity of disintegrated cells of *Agrobacterium rhizogenes* Na, *Agrobacterium rhizogenes* Bi and *Arthrobacter nicotianae*.

Sample code	Incubation time (h)	D-leucine (w/w%)	L-leucine (w/w%)	D-leucine amide (w/w%)
A. nicotianae	139	0.002	0.068	0.56
A. rhizogenes Na	139	0.036	0.078	0.51
A. rhizogenes Bi	139	0.013	0.025	0.61

From the table it can be seen that α-H-α-amino acid amide racemase activity is present in the disintegrated cells of *Agrobacterium rhizogenes* Na, *Agrobacterium rhizogenes* Bi and *Arthrobacter nicotianae* as D-leucine amide is converted into L-leucine; this conversion is not detected in the chemical blanks. In the chemical blanks no D-leucine or L-leucine could be detected even after 139 hours; the D-leucine amide concentration was constant (0.7 w/w%) meaning that D-leucine amide is very stable under the applied reaction conditions.

Example 3

Isolation of the α-H-α-amino acid amide racemase gene from *Ochrobactrum anthropi* IA.

Expression library construction

To obtain single colonies, a glycerol stock of *O. anthropi* IA was streaked onto a yeast carbon base plate containing 0.5% (w/v) DL-α-amino-ε-caprolactam as single nitrogen source, and cultivated at 28°C. A single colony was transferred to 150 ml of LB liquid medium, and grown at 28°C with vigorous shaking to

an $OD_{620\,nm}$ of 0.9. Then cells were harvested by centrifugation and frozen at -20°C.

After thawing the cells, total DNA was isolated according to the Qiagen Genomic DNA Purification Procedure for bacteria using Qiagen Genomic-tip 100/G tips. The standard Qiagen protocol was followed with the following modifications:

- The first incubation step to effect cell lysis was performed without proteinase K for 2 h at 37°C, whereafter twice the suggested amount of proteinase K was added and the solution was incubated for another 2 h at 50°C.
- Before application of the lysate to the Qiagen tips, a centrifugation step (10 min. at 5,000 x g, 4°C) was applied to pellet the particulate matter.
- The lysate from the cells of the 150 ml culture was applied to 3 G/100 columns.

50 µg of the obtained chromosomal DNA was then partially digested with *Sau*3A I at 1/12 U per µg DNA for 30 minutes. Half of the digested DNA was run on a 0.6% agarose gel and DNA fragments between 4 and 10 kb in size were isolated and redissolved in 20 µl of 10 mM Tris-HCl, pH 8.0 buffer.

Vector DNA was prepared by the digestion of 1 µg of pZErO-2 (Invitrogen, Groningen, The Netherlands) with *Bam*H I according to the protocol of Invitrogen.

Linearized vector DNA (50 ng) and *O. anthropi* IA chromosomal DNA fragments (10 µl) were ligated with T4 DNA ligase (according to the protocol of Invitrogen). Subsequently, the ligation mixture was precipitated with NaAc/ethanol and resuspended in 20 µl TE-buffer. 1 µl of this solution was used for the electroporation of electrocompetent *E. coli* DH10B cells (Life Technologies) using a BioRad gene pulser (conditions: 2.5 kV, 25 µF, 100 Ω, 0.1 cm cuvette, 40 µl cell suspension) according to the protocol of the supplier. Transformants were plated onto LB medium with 50 mg/l kanamycin and incubated at 28°C for 24 h. In total over 12,000 colonies were obtained which formed the primary gene library. All 12,000 colonies were pooled in LB medium supplemented with 50 mg/l kanamycin. Part of the cell suspension obtained was used for a total plasmid isolation according to the QiaPrep procedure (Qiagen). This "library in plasmid form" was stored at –20°C till further use. To the remaining part of the cell suspension, glycerol was added to a final concentration of 20% (v/v). The resulting suspension was stored in aliquots at -80°C as primary gene library.

Preparation of L-aminopeptidase help solution

The L-aminopeptidase help solution for the α-H-α-amino acid amide

racemase screening procedure was prepared from a recombinant *E. coli* strain containing plasmid pTrcLAP. The *E. coli* expression vector pTrcLAP contains the *Pseudomonas putida* ATCC 12633 *pep*A gene under the control of the *trc* promoter. Detailed information on this *P. putida* L-aminopeptidase encoding gene can be found in T. Sonke, B. Kaptein, W. H. J. Boesten, Q. B. Broxterman, H. E. Schoemaker, J. Kamphuis, F. Formaggio, C. Toniolo, F. J. T.Rutjes in *Stereoselective Biocatalysis* (Ed.: R. N. Patel). Dekker, New York. 2000, pp. 23-58.

A fresh overnight culture of *E. coli* TOP10 / pTrcLAP was used to inoculate 200 ml of LB medium containing both 0.4 mM of IPTG and 100 mg/l of ampicillin. After overnight growth at 30°C, cells were pelleted by centrifugation and resuspended in 4 ml of 50 mM Tris-HCl, pH 7.5. After cell disruption by one passage through a french press (pressure of 140 Mpa in a 4 ml french press cell), solid particles were collected by centrifugation (45 min. at 40,000 x g, 4°C). The pellet was resuspended in 2 ml of Tris-HCl, pH 7.5 containing 100 mM of $MgSO_4$. This suspension was gently stirred for 30 min. at 4°C. After removal of the particles via centrifugation (45 min. at 40,000 x g, 4°C), the clear solution was stored in aliquots at –20°C for use in the screening assay.

Screening

The "library in plasmid form" solution was diluted 1,000 times in water. 1 µl of this plasmid solution was used to transform electrocompetent *E. coli* TOP10 cells (Invitrogen), and the transformants were plated onto LB medium with 50 mg/l kanamycin. After 2 days of incubation at 28-30°C the obtained colonies were large enough to be transferred to 200 µl of liquid screening medium (tryptone 16 g/l; yeast extract 3 g/l; NaCl 5 g/l; glycerol 2 g/l; pH 7.3) with 50 mg/l kanamycin in microtiter plates. The cultures were grown for 2 days at 28-30°C. Next, a replica of the microtiter plates was prepared by the transfer of a few microliter of each well to new microtiter plates containing solid (0.8% agar) LB medium with 50 mg/l kanamycin. These plates were incubated at 28-30°C for 16-20 h, after which they were stored at 4°C as masterplates. The cells from the remaining part of the cell suspensions were harvested by centrifugation (10 min. at 1,500 x g, room temperature), washed twice with 50 mM Tris-HCl buffer, pH 7.5, and finally re-suspended in 50 µl of 50 mM Tris-HCl buffer, pH 7.5.

The screening reaction was started by the addition of 50 µl of substrate solution containing a mixture of 140 mM D-phenylalanine amide, D-leucine

amide and D-valine amide each in 50 mM Tris-HCl, pH 7.5 and 2 mM $MnCl_2$. After 20 h incubation of the microtiterplates on a shaker at 30°C, 2 µl of L-aminopeptidase help solution (for preparation see earlier section) was added. After an additional 1.5 h incubation at 30°C, all reactions were stopped by the addition of 100 µl of 0.15 M HCl.

Subsequently, the ammonium concentration in all reaction mixtures was determined by transfer of 7 µl of these mixtures to new microtiter plates containing 93 µl of GDH reagent per well. This GDH reagent contained per 100 ml 43 mg of NADH, 116 mg of α-ketoglutaric acid, 11.8 mg of ADP and 1200 U of glutamate dehydrogenase (Sigma) in 150 mM Tris-HCl, pH 8.0. After 15 min. incubation at 37°C the $OD_{340\ nm}$ in each well was measured using a Spectramax plus microtiter plate reader (Molecular Devices, Sunnyvale, California, USA). Clones that showed an $OD_{340\ nm}$ that was lower than the mean value of the microtiter plate containing this clone decreased with three times the standard deviation of that same microtiter plate, were regarded as potential positive clones.

Of 11,272 clones screened, 32 clones could be identified as potential positive clones.

Confirmation of potential positive clones

Of all 32 potential positive clones identified in the screening, material from the masterplates was used to inoculate 3 ml of liquid screening medium containing 50 mg/l kanamycin. After growth for 2 days at 30°C, cells were collected by centrifuging 2 ml of these cell cultures. Then the cell pellets were washed four times in 25 mM Tris-HCl buffer, pH 7.5. Subsequently, the cell pellets were resuspended in 100 µl substrate solution containing 70 mM of D-phenylalanine amide, or D-leucine amide, or D-valine amide in 50 mM Tris-HCl, pH 7.5 and 1 mM $MnCl_2$. After incubation for 20 h at 30°C, the reactions were stopped by the addition of 100 µl of 0.15 M HCl, after which the reaction mixtures were analyzed by the HPLC method described in example 1.

Of the 32 potential positive clones identified in the screening, one showed significant formation of L-leucine from D-leucine amide. This clone contained the *O. anthropi* IA α-H-α-amino acid amide racemase gene on its 7.7 kb plasmid. This was concluded from the fact that transformation of this plasmid into *E. coli* TOP10 cells inevitably led to recombinant cells that converted D-leucine amide into L-Leucine. Sequencing of this plasmid, that was named pOa(1)PLV49B10, revealed the complete nucleotide sequence of the α-H-α-amino acid amide racemase gene. The sequence of

this gene is listed as nucleotides 98 to 1417 (including the TAA stopcodon) of SEQ ID: NO. 1 encoding the protein of SEQ ID: NO. 2 as presented *infra*.

Example 3A:

Isolation of the α-H-α-amino acid amide racemase gene from *Arthrobacter nicotianae* NCIMB 41126

Expression library construction

A glycerol stock of *Arthrobacter nicotianae* NCIMB 41126 was plated onto a yeast carbon base plate containing 0,2% (w/v) D-α-amino-ε-caprolactam as sole N-source and cultivated for 3-4 days at 28°C to get isolated colonies of the desired size. A single colony was transferred to 50 ml LB medium, and grown overnight at 28°C under vigorous shaking. During the last half hour of this growth period, carbenicillin (final concentration 200 µg/ml) was added to weaken the cell wall. The cells were harvested by centrifugation, and the pellet obtained was re-suspended in 5 ml of 50 mM Tris-HCl, pH 8.0 containing 50 mM EDTA. After addition of 100 µl of lysozyme (100 mg/ml) and 25 µl of proteinase K (20 mg/ml) the suspension was incubated for 30 minutes at 37°C. Then 6 ml of Nuclei Lysis Solution (Promega Corporation, Madison, USA) was added followed by an incubation for 15 minutes at 80°C. Finally, the solution was incubated at 65°C till complete lysis. After RNase treatment (final concentration 4 µg/ml) for 30 minutes at 37°C, 2 ml Protein Precipitation Solution (Promega Corporation, Madison, USA) was added, and the solution was vortexed for 20 seconds followed by incubation on ice. After centrifugation (10'; 4,500 x g, 4°C), the supernatant was transferred to a mixture of 0.1 volumes of NaAc (3M, pH 5) and 2 volumes of absolute ethanol. The precipitated genomic DNA was hooked to a sterile pipette and transferred to a tube with 2 ml of 10 mM Tris-HCl (pH 8). After the gDNA had dissolved, measurement of the A_{260nm} and A_{280nm} showed that about 2.3 mg of good quality gDNA had been isolated. Agarose gel electrophoresis proved that the gDNA isolated did not contain rRNA and that its size was larger than 15 kb.

50 µg of the gDNA was partially digested with *Sau*3A I at 1/24 U per µg of DNA at 37°C for 30 minutes. Part of the digested DNA was run on a preparative 0.6% agarose gel and DNA fragments between 4 and 10 kb in size were isolated from the gel using the QIAquick extraction kit (Qiagen), concentrated by NaAc/ethanol precipitation and redissolved in 10 µl of 10 mM Tris-HCl, pH 8.0 buffer.

Vector DNA was prepared by digestion of 1 µg of pZErO-2 (Invitrogen, Breda, The Netherlands) with *Bam*H I according to the protocol of the supplier.

Linearized vector DNA (10 ng) and *A. nicotianae* NCIMB 41126 gDNA fragments (2.5 µl) were ligated in a total volume of 10 µl with 2,5 U of T4 DNA ligase for 1 hour at 16°C. Subsequently, the ligation mixture was precipitated with NaAc/ethanol and resuspended in 5 µl of TE-buffer. 2.5 µl of this solution (about 5 ng of vector) was used for the electroporation of electrocompetent *E. coli* DH10B cells (Life Technologies) using a BioRad gene pulser (conditions: 2.5 kV, 25 µF, 100 Ω, 0.1 cm cuvette, 40 µl cell suspension) according to the protocol of the supplier. Transformants were plated onto LB medium with 50 mg/l kanamycin and incubated at 28°C for 24 h. In total about 100,000 colonies were obtained which formed the primary gene library. All 100,000 colonies were pooled in LB medium supplemented with 50 mg/l kanamycin. Part of this cell suspension was used for a total plasmid isolation according to the QiaPrep procedure (Qiagen). This "library in plasmid form" was stored at -20°C till further use. To the remaining part of the cell suspension, glycerol was added to a final concentration of 8% (v/v). The resulting suspension was stored in aliquots at -80°C as primary gene library.

Screening

The *A. nicotianae* NCIMB 41126 library was screened for α-H-α-amino acid amide racemase containing clones using the protocol given in example 3, however with a slightly modified substrate solution. Besides all components mentioned in example 3, the substrate solution in this screening also contained 20 µM of pyridoxal-5-phosphate.

In total 10,691 clones of the *A. nicotianae* NCIMB 41126 library were screened. 2 of these clones were identified as potential positive clones.

Confirmation of potential positive clones

Confirmation of both potential positive clones was done according to the protocol given in example 3, with the exception that all 3 substrate solutions used contained 10 µM of pyridoxal-5-phosphate.

This experiment demonstrated that both clones significantly converted D-leucine amide into L-leucine and D-valine amide into L-valine. As

indicated in example 1, these conversions proved the presence of α-H-α-amino acid amide racemase activity in these clones. Retransformation experiments in which the plasmids from both clones were introduced into *E. coli* TOP10 cells, showed that this α-H-α-amino acid amide racemase activity is plasmid encoded, because all recombinant cells tested converted D-leucine amide into L-leucine and D-valine amide into L-valine.

The plasmids from both positive clones were then subjected to restriction enzyme analysis. This analysis clearly showed that both recombinant plasmids contained an overlapping insert. Therefore, only one of these plasmids, i.e. pAn(1)PLV36D6, was sequenced. Analysis of the nucleotide sequence of this plasmid with a total size of 7.1 kb, revealed the complete nucleotide sequence of the α-H-α-amino acid amide racemase gene. The sequence of this gene is listed as nucleotides 80 to 1417 (including the TGA stop codon) of SEQ ID: No. 8 encoding the protein of SEQ ID: No. 9 as presented *infra*. As is shown below, it is also possible to express active α-H-α-amino acid amide racemase starting from nucleotide position 41 (GTG) from SEQ ID: NO. 8.

<u>Example 4</u>

<u>Construction of plasmid pKEC-AZAR</u>

The *O. anthropi* IA α-H-α-amino acid amide racemase gene was subcloned into *E. coli* expression vector pKECaroP using PCR. Expression vector pKECaroP is similar to construct pKECtrp, whose construction has been described in WO 00/66751, except that pKECaroP contains the pSC101 derived par function and the *E. coli aro*H promoter instead of the *trp* promoter. The α-H-α-amino acid amino racemase open reading frame was amplified using

5'- GCCTCA<u>CATATG</u>CAAACACCGCTTTCATTGCG - 3' [SEQ ID: NO. 6]

as forward primer (with *Nde* I cleavage site underlined), and

5'- GCCTCA<u>CCCGGG</u>TTACCACATCATAAAATGGGCGACATC - 3' [SEQ ID: NO. 7] as reverse primer (with *Xma* I cleavage site underlined), and plasmid pOa(1)PLV49B10 as template. This PCR, that was performed with PCR SuperMix High Fidelity (Life Technologies) according to the supplier's protocol, yielded a single fragment. Correct size (1,341 bp) of the amplified fragment was confirmed by agarose gel electrophoresis.

After purification of the amplified fragment with the QIAquick PCR

Purification Kit (Qiagen), the fragment was cloned into vector pCR®4Blunt-TOPO (Invitrogen). The cloning mix was subsequently used to transform One Shot™ Chemically Competent *E. coli* TOP10 Cells (Invitrogen). Recombinant cells were selected by plating the whole transformation mixture on LB plates containing 100 µg/ml carbenicillin, followed by overnight incubation at 28°C.

After overnight cultivation of material from six colonies in 5 ml LB medium containing 100 µg/ml carbenicillin, plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Digestion with restriction enzymes *Nde* I and *Xma* I proved that five out of these six colonies contained the desired recombinant vector.

Plasmid DNA of the five correct clones was pooled and digested to completion with *Nde* I and *Xma* I. The total digestion mixture was applied to a preparative 1% agarose gel for separation of the different fragments. The correct fragment (1,322 bp) was subsequently isolated from the gel by the QIAquick Gel Extraction Kit (Qiagen) and stored at –20°C till further use as insert fragment.

Plasmid pKECaroP was digested to completion with *Nde* I and *Xma* I, yielding two fragments of 2,850 and 3,036 bp as shown by analytical agarose gel electrophoresis. After heat inactivation of the *Nde* I and *Xma* I restriction enzymes (20 minutes, 65°C), *Bsa* I was added to the mixture to cut the undesired 2,850 bp fragment into two smaller pieces. The complete digestion mixture was loaded on a 1% preparative agarose gel, followed by isolation of the desired 3,036 bp fragment using the QIAquick Gel Extraction Kit (Qiagen), that was stored at –20°C till further use as vector fragment.

Vector and insert fragment were ligated using T4 DNA Ligase and the resulting ligation mixture was used for transformation of One Shot™ Chemically Competent *E. coli* TOP10 Cells. The transformation mix was plated on LB plates containing 50 µg/ml kanamycin, that were incubated at 28°C till sufficiently large colonies appeared.

Colony-PCR using PCR SuperMix (Life Technologies) and the above given primers ([SEQ ID: NO. 6] and [SEQ ID: NO. 7]) was performed to screen for colonies containing the correct recombinant vector. Material from twelve PCR positives was used to inoculate 12 tubes with 5 ml LB medium containing 50 µg/ml kanamycin. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit. Digestion with restriction enzyme *Hind* III yielded two fragments of 1,731 and 2,627 bp with all twelve plasmids, proving that all twelve colonies contained the desired recombinant vector.

Five of these twelve positive clones were cultured in 25 ml LB medium supplemented with 50 µg/ml kanamycin. After overnight incubation at 28°C, cells were harvested via centrifugation and washed in the same sonification buffer as used in example 2. After resuspending the cell pellets in sonification buffer (ratio wet weight cells to sonification buffer 1:10), the cells were disintegrated by sonification. Crude cell extracts were obtained by removal of the particulates via centrifugation.

The five obtained crude cell extracts were tested for α-H-α-amino acid amide racemase activity by mixing 0.5 ml thereof with 4.5 ml substrate solution consisting of a 22.2 mM HEPES-NaOH buffer pH 7.7 containing 0.01 mM of pyridoxal-5-phosphate and 66 mM D-leucine amide. Reactions were incubated at room temperature. Samples were taken after 0, 18 and 44 hours that were transferred to an equal volume of 1 M H_3PO_4 to stop the reaction. These samples were analyzed by HPLC according to the method given in example 1.

With the crude extract of two of the five clones, called *E. coli*/pKEC_AZAR_3 and *E. coli*/pKEC_AZAR_11, significantly more L-leucine and L-leucine amide was detected in the 18 and 44 hour samples than in the 0 hour sample. Finally, nucleotide sequencing of pKEC_AZAR_3 and pKEC_AZAR_11 revealed the correct nucleotide sequence of the α-H-α-amino acid amide racemase gene and flanking vector parts in these two recombinant plasmids.

Example 4A

Construction of plasmid pBADAn36D6-DEST

The *A. nicotianae* NCIMB 41126 α-H-α-amino acid amide racemase gene was subcloned into *E. coli* expression vector pBAD/*Myc*-His-DEST using PCR and GATEWAY Cloning Technology (Invitrogen). The α-H-α-amino acid amide racemase open reading frame was first PCR amplified using

5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGAGGAATTAACC*ATG*-AGTACGCCGCGTTGCGGGGAG -3' [SEQ ID: No. 10]

as forward primer (with Shine-Delgarno site underlined, ATG startcodon in italic and *attB1* site double underlined), and

5'- GGGGACCACTTTGTACAAGAAAGCTGGGT*TCA*CCAACCAGCATAGG-GAGCGATCTG -3' [SEQ ID: No. 11]

as reverse primer (with stop codon in italic and *attB2* site double underlined), and plasmid pAn(1)PLV36D6 as template. The identification of plasmid pAn(1)PLV36D6

has been described in example 3A. The PCR, which was performed with Expand High Fidelity polymerase (Roche Applied Science, Mannheim, Germany) according to the supplier's protocol, yielded a single fragment. Correct size (1,449 bp) of the amplified fragment was confirmed by agarose gel electrophoresis.

After purification of the amplified fragment from a preparative agarose gel with the QIAquick Gel Extraction Kit (Qiagen), the fragment was used as a substrate for the so-called BP *in-vitro* recombination reaction, which was performed according to the GATEWAY manual of the supplier (Invitrogen). Recombination between the *attB*-PCR fragment and the pDONR207 Donor Vector and subsequent transformation of the obtained mixture into *E. coli* DH5α competent cells (Invitrogen) resulted in the ENTRY clone pAn36D6-ENTRY. Recombinant cells were selected by plating the whole transformation mixture on 2*TY plates containing 7 μg/ml of gentamicin, followed by overnight incubation at 37°C. Plasmid DNA was isolated from a few single colonies using the QIAprep Spin Miniprep Kit (Qiagen), after having cultivated them in 2*TY medium containing 7 μg/ml of gentamicin for 16 hours. Digestion with restriction enzymes *Pvu* II, *Nsp* I (New England Biolabs, Frankfurt, Germany) and *Bss*S I (New England Biolabs, Frankfurt, Germany), respectively, proved that all tested colonies contained the desired recombinant vector. Then, the *attB*-PCR inserts of four of these correct clones were sequenced. All four pAn36D6-ENTRY plasmids sequenced proved to contain the sequence as expected based on SEQ ID: No. 8 and the two PCR primers used.

Subsequently the α-H-α-amino acid amide racemase containing PCR fragment was introduced in Destination vector pBAD/*Myc*-His-DEST (*vide infra*) via the so-called LR *in-vitro* recombination reaction using one of the correct pAn36D6-ENTRY clones and pBAD/*Myc*-His-DEST. Also this reaction was performed according to the supplier's procedure. The recombination mix was used to transform One Shot™ Chemically Competent *E. coli* TOP10 Cells (Invitrogen). Recombinant cells were selected by plating the whole transformation mixture on 2*TY plates containing 100 μg/ml ampicillin followed by overnight incubation at 37°C. After overnight cultivation of a few colonies in 2*TY medium containing 100 μg/ml ampicillin, plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Digestion with restriction enzymes *Acc* I and *Rsr* II (New England Biolabs, Frankfurt, Germany), respectively, proved that all colonies contained the desired recombinant vector pBADAn36D6-DEST.

A single colony of strain *E. coli* TOP10/pBADAn36D6-DEST was

used to inoculate 50 ml 2 x TY medium supplemented with 100 µg/ml carbenicillin. After overnight incubation at 28°C, 500 µl of this culture was used to inoculate 500 ml 2*TY medium supplemented with 100 µg/ml carbenicillin and 0,005 % arabinose. After overnight incubation at 28°C, cells were harvested via centrifugation (15 min. at 6,200 x g, 4°C) and washed in a solution containing 20 mM HEPES-NaOH, pH 7.5, 20 µM pyridoxal-5-phosphate, and 1.3 mM of dithiothreitol. After resuspending the cell pellets in the same buffer buffer solution (ratio wet weight cells to sonification buffer 1:3), the cells were disintegrated by 10 min. sonification with a Soniprep 150 of MSE operated at maximum amplitude using time intervals of 10 sec. (i.e. 10 sec. sonification, 10 sec. cooling) and cooling in ice/acetone. Crude cell extracts were obtained by removal of the particulates via centrifugation. Finally, sucrose was added to the cell free extract to a final concentration of 0.25 M.

The cell extract was tested for racemase activity by mixing 0.5 ml thereof with 9.5 ml substrate solution. Reactions were incubated at 37 °C. Samples were taken at regular time intervals, that were transferred to an equal volume of 1 M H_3PO_4 to stop the reaction. These samples were analyzed by HPLC according to the method given in example 1.

This experiment showed that the substrate was entirely racemized within 20 minutes. Therefore, it is concluded that the *A. nicotianae* NCIMB 41126 α-H-α-amino acid amide racemase is encoded by the open reading frame depicted in SEQ ID: No. 8.

Example 4B

Construction of GATEWAY Destination vector pBAD/*Myc*-His-DEST

Destination vector pBAD/*Myc*-His-DEST, that was used for the expression of the α-H-α-amino acid amide racemase gene from *A. nicotianae* NCIMB 41126 in *E. coli* was prepared by introducing a cat/ccdB cassette into the commercially available *E. coli* expression vector pBAD/Myc-HisC. The cat/ccdB cassette was amplified by PCR using

5'- AAGAAGACCGGATCCTACCTGACGCTTTTTATCGCAACTCTC-TACTGTTTCTCCATACCCGTTTTTTGGGCTAAC*ACAAGTTTGT-ACAAAAAAGC*TGAAC -3' [SEQ ID: No. 12]

as forward primer (with promoter sequence double underlined, *Bpl* I recognition and cleavage site underlined and *att*R sequences in italic), and

5'- TTGTTC<u>TACGTA</u>*ACCACTTTGTACAAGAAAG*CTGAAC -3' [SEQ ID. No. 13] as reverse primer (with *Sna*B I cleavage site underlined and *att*R sequences in italic), and vector pDEST15 (Invitrogen) as template. The PCR, which was performed with Expand High Fidelity polymerase (Roche Applied Science, Mannheim, Germany) according to the supplier's protocol, yielded a single fragment. Correct size (1792 bp) of the amplified fragment was confirmed by agarose gel electrophoresis. After purification of the amplified fragment from a preparative agarose gel with the QIAquick Gel Extraction Kit (Qiagen), the fragment was digested to completion with *Bpi* I (MBI Fermentas, St.Leon-Rot, Germany) (resulting in a overhang complementary to *Bam*H I) and *Sna*B I (New England Biolabs, Frankfurt, Germany) and ligated with T4 DNA ligase into the *E. coli* expression vector pBAD/*Myc*-HisC (Invitrogen), which had been digested with *Bam*H I and *Sna*B I. The ligation mix was subsequently used to transform Chemically Competent *E. coli* DB3.1 cells (Invitrogen). Recombinant cells were selected by plating the whole transformation mixture on 2*TY plates containing 35 µg/ml chloramphenicol followed by overnight incubation at 37°C. After isolation of the recombinant plasmid from three individual colonies, the inserts were sequenced. One of these clones proved to contain the desired insert, and was named pBAD/*Myc*-His-DEST. Although 7 abbreviations were observed between the nucleotide sequence of the sequenced part of plasmid pBAD/*Myc*-His-DEST and the reference sequence (Invitrogen – nucleotide sequence of pDEST15), all the essential features (chloramphenicol resistance, *ccd*B selection and *att*R recombination) of pBAD/*Myc*-His-DEST were fully functional.

<u>Example 5</u>

<u>α-H-α-Amino acid amide racemase activity test using D-leucine amide as substrate and whole *E. coli* cells containing the α-H-α-amino acid amide racemase gene from *Ochrobactrum anthropi* IA as biocatalyst</u>

Preparation of the cells

A single colony of *E. coli* DH10B containing plasmid pOA(1)PLV49B10 was transferred to LB medium with kanamycin (50 mg/l) to prepare a pre-culture. After overnight incubation at 28°C and 200 rpm, this pre-culture was used to inoculated a flask with 500 ml of 2*TY medium (10 g/l Yeast extract, 16 g/l Tryptone, 5 g/l NaCl) containing kanamycin (50 mg/l). After overnight growth at 28°C (OD_{620nm} =

4.4) and 200 rpm, cells were harvested by centrifugation (15 min. at 6,200 x g, 4°C), and washed with 50 mM HEPES-NaOH buffer of pH 7.7.

To be able to determine the background activity of the *E. coli* host-vector system in the activity tests, an *E. coli* DH10B strain containing a pZErO-2 based construct with a mutant Green Fluorescent Protein (GFPuv) encoding gene as insert in the opposite direction as the vector borne *lac* promoter, was cultivated via the same procedure (*E. coli* DH10B / pZErO-GFPuv-wrong-orientation). Growth of this recombinant *E. coli* strain resulted in an overnight culture with an OD_{620nm} of 3.8.

The cell pellets from both cultures were subsequently washed and resuspended in 25 ml of 50 mM HEPES-NaOH buffer, pH 7.7. Aliquots of 2 ml of both cell suspensions were centrifuged once more and the pellets were stored at -20°C until execution of the activity tests described below. The remaining part of these cell suspensions was stored at -20°C for use in example 6.

α-H-α-amino acid amide racemase activity towards D-leucine amide

To determine the activity of *E. coli* / pOA(1)PLV49B10 and the *E. coli* control, the above-mentioned cell pellets were thawed and resuspended to a total volume of 1 ml with 50 mM HEPES-NaOH buffer, pH 7.7. Then, reaction mixtures of 10 ml each were prepared containing 50 mM HEPES-NaOH buffer (pH 7.7), 10 μM pyridoxal-5-phosphate (PLP), 2.5 wt% of D-leucine amide, if applicable 20 mM of EDTA to suppress the *E. coli* amidase activity, and 0.5 ml of the *E. coli* / pOA(1)PLV49B10 and *E. coli* DH10B / pZErO-GFPuv-wrong-orientation cell suspensions or water (for chemical blanks). Reactions were started by the addition of the cells. Reaction mixtures were incubated at 30°C and 175 rpm. Directly after addition of the cells (t = 0 hours) and after 27 and 43 hours samples were taken in which the reaction was stopped by removal of the cells via centrifugation followed by 0.22 μM filtration. Finally, the filtered samples were stored at –20°C until analysis by chiral HPLC as described below:

column:	Sumichiral OA5000 from Sumika (150 x 4.6 mm I.D., 5μ) + guard column
eluent:	85 v/v% 2mM $CuSO_4$ + 15 v/v% methanol
flow:	1.0 ml/min.
column temp.:	40°C
inj. volume:	5μl

detection: fluorescence detection after post-column reaction with *o*-phthalaldehyde and 2-mercaptoethanol (wavelength ex=338 nm and em>420 nm)

The results of this experiment are presented in table 4.

Table 4. α-H-α-amino acid amide racemase activity towards D-Leucine amide of *E. coli* DH10B / pOA(1)PLV49B10 and *E. coli* blank (*E. coli* DH10B / pZErO-GFPuv-wrong-orientation).

Strain	20 mM EDTA	Incubation time (h)	D-leucine amide (wt%)	L-leucine amide (wt%)	D-leucine (wt%)	L-leucine (wt%)
B	-	0	2.67	n.d.	n.d.	n.d.
B	-	27	2.51	n.d.	0.003	0.005
B	-	43	2.63	0.060	0.005	0.004
A	-	0	2.63	n.d.	n.d.	n.d.
A	-	27	1.03	0.170	0.009	1.71
A	-	43	0.512	0.063	0.017	2.12
A	+	0	2.61	n.d.	n.d.	n.d.
A	+	27	1.77	0.555	0.003	0.313
A	+	43	1.59	0.772	0.006	0.345

n.d.: Not detectable

Strain A: *E. coli* DH10B / pOA(1)PLV49B10
Strain B: *E. coli* DH10B / pZErO-GFPuv-wrong-orientation

The data in table 4 clearly show that *E. coli* DH10B / pZErO-GFPuv-wrong-orientation could not convert D-leucine amide. Even after 43 hours of reaction, the reaction mixture contained the same amount of D-leucine amide as at the start of the reaction. This was also the case for all chemical blanks (data not shown).

With *E. coli* DH10B / pOA(1)PLV49B10 cells on the other hand, the amount of D-leucine amide clearly decreased in time. Without additional EDTA, this substrate was converted to a relatively low amount of L-leucine amide and a large amount of L-leucine. With additional EDTA, a much higher concentration of L-leucine amide was obtained, because EDTA, a chelating compound partially inhibits the amidase activity of *E. coli*, thereby reducing the conversion of L-leucine amide to

L-leucine.

The results obtained in this experiment prove that *E. coli* DH10B / pOA(1)PLV49B10 cells contain α-H-α-amino acid amide racemase activity towards D-leucine amide. Furthermore, they show that by combining this α-H-α-amino acid amide racemase with an L-selective amidase / aminopeptidase (as present in e.g. *E. coli* DH10B), D-α-H-α-amino acid amides can be converted to L-α-H-α-amino acids.

<u>Example 6</u>

<u>α-H-α-Amino acid amide racemase activity test using DL-leucine amide as substrate and whole *E. coli* cells containing the α-H-α-amino acid amide racemase gene from *Ochrobactrum anthropi* IA as biocatalyst</u>

The *E. coli* DH10B / pOA(1)PLV49B10 and *E. coli* DH10B / pZErO-GFPuv-wrong-orientation cell suspensions from example 5 (Preparation of the cells) were used. To determine their activity towards racemic DL-leucine amide, identical reaction mixtures were prepared as in example 5, except that 2.5 wt% of DL-leucine amide was used, EDTA was ommitted in all reactions, and the reactions were started with 2 ml of the cell suspensions. Samples were taken directly after the start of the reactions (t = 0 hours) and after 8 and 24 hours.

The results of this experiment are presented in table 5.

Table 5. α-H-α-amino acid amide racemase activity towards DL-leucine amide of *E. coli* DH10B / pOA(1)PLV49B10 and *E. coli* blank (*E. coli* DH10B / pZErO-GFPuv-wrong-orientation).

Sample code	Incubation time (h)	D-leucine amide (wt%)	L-leucine amide (wt%)	D-leucine (wt%)	L-leucine (wt%)	e.e.[a] L-leucine (%)
B	0	1.13	1.15	0.001	0.004	-
B	8	1.15	0.12	0.001	1.43	99.9
B	24	1.23	n.d.	0.002	1.48	99.7
A	0	1.19	1.25	0.001	0.003	-
A	8	1.24	0.33	0.004	1.45	99.4
A	24	0.43	0.14	0.028	2.25	97.5

n.d. : Not detectable

[a] The e.e. (enantiomeric excess) of the L-acid was calculated using the formula:

$e.e._{L\text{-}acid} = [(L\text{-}acid - D\text{-}acid) / (L\text{-}acid + D\text{-}acid)] \times 100\%$

Strain A: *E. coli* DH10B / pOA(1)PLV49B10

Strain B: *E. coli* DH10B / pZErO-GFPuv-wrong-orientation

From the data in table 5 it becomes clear that the *E. coli* blank cells (*E. coli* DH10B / pZErO-GFPuv-wrong-orientation) can only convert L-leucine amide to L-leucine with an enantiomeric excess of over 95%. The D-leucine amide is left untouched, thereby resulting in a maximum yield of 50% only.

With the *E. coli* DH10B / pOA(1)PLV49B10 cells containing the α-H-α-amino acid amide racemase encoding gene, clearly both L- and D-leucine amide are converted to L-leucine, leading to a yield of over 50% and maximum 100%. Again, the enantiomeric excess of the obtained L-leucine is well over 95%.

This experiment proves that by combining the α-H-α-amino acid amide racemase from *O. anthropi* IA with an L-selective amidase / aminopeptidase (as present in e.g. *E. coli* DH10B), DL-α-H-α-amino acid amides can be converted into L-α-H-α-amino acids in a more than 50% yield.

Example 7

Determination of the substrate specificity of the α-H-α-amino acid amide racemase from *Ochrobactrum anthropi* IA using cell free extract from *E. coli* TOP10 / pKEC_AZAR_3

Preparation of the cell free extract

Two pre-cultures of *E. coli* TOP10 / pKEC_AZAR_3 (see example 4) were prepared by inoculation of 2 erlenmeyer flasks with 100 ml of 2*TY medium (10 g/l Yeast extract, 16 g/l Tryptone, 5 g/l NaCl) containing 50 mg/l of kanamycin with 10µl of a glycerol stock per flask. After overnight cultivation at 28°C and 200 rpm, the pre-cultures were pooled, and subsequently used to inoculate 2 erlenmeyer flasks each containing 1 l of the same medium. Also these flasks were cultivated at 28°C and 200 rpm for 16-18 h. Cells were harvested by centrifugation (15 min. at 6,200 x g, 4°C), washed with 20 mM HEPES-NaOH, pH 7.5, pooled, weighed, and resuspended in a solution containing 20 mM HEPES-NaOH, pH 7.5, 20 µM pyridoxal-5-phosphate, and 1.3 mM of dithiothreitol in a 1:3 ratio of wet weight cells to solution. Then, cells were disintegrated by 10 min. sonification with a Soniprep 150 of MSE operated at maximum amplitude using time intervals of 10 sec. (i.e. 10 sec. sonification, 10 sec. cooling) and cooling in ice/acetone. Particles were removed by centrifugation, after which sucrose was added to the cell free extract to a final concentration of 0.25 M. The resulting cell free extract (CFE) was stored in aliquots at -20°C. *E. coli* TOP10 / pBAD/*Myc*-HisC (Invitrogen) cells were treated in exactly the same way and served as negative control.

Determination of the substrate specificity

The substrate specificity of the α-H-α-amino acid amide racemase from *O. anthropi* IA was determined by measuring the racemase activity of the above-mentioned CFE towards a number of enantiomerically pure amino acid amides. Each reaction mixture of 10 ml (including the CFE) contained 50 mM HEPES-NaOH, pH 8.0, 20 µM pyridoxal-5-phosphate, 0,4 mg/ml BSA, 1 mM dithiothreitol, 20 mM EDTA and 75 mM of one of the amino acid amides tested. Reactions were started by the addition of 1 ml of the thawed CFE of *E. coli* TOP10 / pKEC_AZAR_3 or *E. coli* TOP10 / pBAD/*Myc*-HisC (blank) and were incubated at 37°C. At regular time intervals samples of 1 ml were taken and transferred to vials containing 1 ml of 1M H_3PO_4 to stop the reaction. After sufficient dilution of the samples in 0.4 M borate buffer pH 9.4, the mixtures obtained were analyzed using HPLC to determine the exact concentrations of

the two amino acid and two amino acid amide enantiomers according to the following method.

column: Inertsil ODS-3 150 x 4.6 mm I.D.

eluent A: 50 mM sodium acetate, pH 6.0 with acetic acid/ acetonitrile 95/5 v/v%

eluent B: 50 mM sodium acetate, pH 6.0 with acetic acid/ acetonitrile 30/70 v/v%

gradient:

Time (min)	Eluent A (v/v%)	Eluent B (v/v%)
0 100	0	
28	0	100
33	0	100
33.1	100	0
41 (stoptime)		

flow: 1.0 ml/min.

column temp.: 40°C

inj. volume: 20 µl

detection: fluorescence detection after pre-column reaction with o-phthaldehyde and D-3-mercapto-2-methylpropionic acid. (wavelength ex=338 nm and em>420 nm)

Pre-column reagents:

Reagent A: 50 mM Ortho Phtalic Anhydride (170 mg/25 ml MeOH/water 50/50

Reagent B: 113 mg D-*S*-benzoyl-3-mercapto-2-methylpropionic acid in 1 ml of 1 M NaOH and dissolve. Add 9 ml of MeOH/water 50/50 and adjust to pH 7.0 with 1 M H_3PO_4

Reagent C: 0.25 M H_3PO_4

Derivatization procedure:

35 µl Reagent A

35 µl Reagent B

210 µl sample solution (sample in 0.4 M borate buffer pH 9.4)

140 µl Reagent C

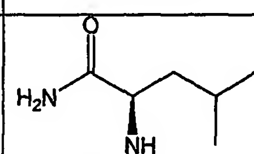
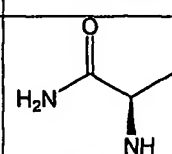
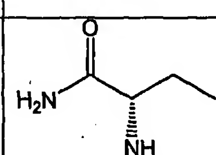
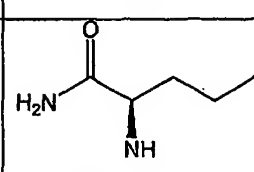
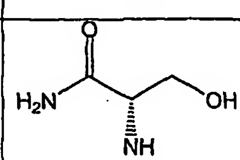
Reaction time: 5 minutes

The initial activity for the different substrates tested was calculated from the linear parts of the progress curves, in which the conversion was plotted against the reaction time. Use of the linear part of the progress curves secured that

only samples taken at time points at which the substrate concentration was still sufficiently high, were used for the calculations. The conversion was calculated by dividing the amount of amino acid and amino acid amide formed via the racemase reaction (so with the opposite stereochemistry compared to the substrate molecule) by the total amount of the two amino acid enantiomers and the two amino acid amide enantiomers. In this way, the calculated conversions were corrected for potential hydrolysis of the amide substrate and/or product by amidases / aminopeptidases that were not completely inhibited by the EDTA in the reaction mixture.

With the CFE of *E. coli* TOP10 / pBAD/*Myc*-HisC none of the enantiopure α-H-α-amino acid amide substrates used was racemized. *E. coli* TOP10 clearly doesn't contain an α-H-α-amino acid amide racemase. The results of this experiment with the CFE from *E. coli* TOP10 / pKEC_AZAR_3 are given in table 6.

Table 6. Relative initial activity of CFE from *E. coli* TOP10 / pKEC_AZAR_3 towards different enantiomerically pure α-H-α-amino acid amides

Substrate	Structure	Rel. initial activity (%)[a]
D-leucine amide		100
D-alanine amide		85
L-2-aminobutyric acid amide		203
D-norvaline amide		94
L-serine amide		83

[a] All initial activities are given relative to the activity towards D-leucine amide

The results presented in table 6 clearly show that the CFE from *E. coli* TOP10 / pKEC_AZAR_3 can not only racemize leucine amide, but also other α-H-α-amino acid amides. Because this recombinant strain contains the α-H-α-amino acid amide racemase gene from *O. anthropi* IA, this broad substrate specificity can only be attributed to the racemase encoded by this gene.

Example 8

Determination of the substrate specificity of the partially purified α-H-α-amino acid amide racemase from *Ochrobactrum anthropi* IA

Partial purification

The α-H-α-amino acid amide racemase from *O. anthropi* IA was partially purified from *E. coli* DH10B / pOa(1)PLV49B10. To obtain sufficient cell material for this purification, this recombinant strain was fermented at 10 liter scale. First, a seed culture was prepared by the inoculation of 500 ml of 2*TY medium containing 50 mg/l of kanamycin with 10 µl of a glycerol stock of this strain. After 22 hours of incubation at 28°C and 170 rpm, an $OD_{620\ nm}$ of 2.2 was obtained. The complete seed medium was then transferred into a fermentor (Type ISF-200, Infors, Bottmingen, Switzerland) containing 9 liter of 2*TY medium containing 50 mg/l of kanamycin. pH was controlled at 7.0 by the addition of 5 N of H_3PO_4 and/or 5 N KOH, and the pO_2 was manually adjusted by changing both the aeration (1 - 2 liter of air per minute) and stirrer speed (250 – 750 rpm). The fermentor was operated at 28°C for 19 hours, resulting in a cell suspension with an $OD_{620\ nm}$ of 5.7. The cells were harvested by centrifugation (15 min. at 12,000 x g, 4°C) , washed once in 20 mM HEPES-NaOH, pH 7 and stored as wet cell pellet (about 125 g) at -20°C.

After thawing the cell pellet, cells were resuspended in buffer A (20 mM HEPES-NaOH, pH 7.5, 1.3 mM dithiothreitol, 20 µM pyridoxal-5-phosphate) containing 2.66 mM $MgCl_2$. Per gram of wet weight cells, 3 grams of buffer A were used. Just before disintegration, benzonase (30 U/gram of wet weight cells - Merck KGaA, Darmstadt, Germany) was added to the cell suspension. Cells were disintegrated by one passage through a homogenizer (Haskel, model NJ1600HD15, Wesel, Germany) at an operating pressure of 1,500 bar. Then particles were removed by centrifugation (30 min. at 34,000 x g, 4°C) and the resulting cell free extract (CFE)

was stored in aliquots at -20°C.

All following purification steps were executed at 4°C. The first purification step was an ammonium sulphate precipitation at 40% saturation. This step was executed by adding an 80% saturated $(NH_4)_2SO_4$ solution drop by drop to an equal volume of CFE. The turbid solution was slowly shaken for 1.5 h, after which the precipitated protein was collected by centrifugation (30 min. at 15,000 x g). The protein pellet was dissolved in buffer A, and used in the next purification step.

To enable anion exchange chromatography as second purification step, the protein solution after ammonium sulphate precipitation had to be desalted. Therefore, it was applied in 2.5 ml portions to PD-10 desalting columns (Amersham Biosciences, Roosendaal, The Netherlands), that had been equilibrated with 20 mM Tris-HCl, pH 8.0 (buffer B). Elution of the protein was effected with 3.5 ml of buffer B per PD-10 column. The desalted protein solution was applied to a Mono Q HR 10/10 column (Amersham Biosciences, Roosendaal, The Netherlands) that had been equilibrated with buffer B. This column was operated at a flow rate of 4 ml/min, and fractions of 4 ml were collected. The α-H-α-amino acid amide racemase was eluted from this column with buffer B containing 1 M NaCl applying a linear gradient of 0 – 100% NaCl in 100 ml. The α-H-α-amino acid amide racemase eluted at about 0.34 M NaCl and the active fractions were pooled.

The α-H-α-amino acid amide racemase containing protein solution was then applied to a HiLoad 26/60 Superdex 200 prep grade gel filtration column (Amersham Biosciences, Roosendaal, The Netherlands) that had been equilibrated with buffer B containing 1 M NaCl. The α-H-α-amino acid amide racemase was eluted with the equilibration buffer at a flow rate of 3 ml/min. Fractions of 3 ml were collected. The fractions containing the racemase activity were pooled and sucrose was added to a concentration of 0.25 M before storage in aliquots at -20°C.

Using this purification protocol, the *O. anthropi* IA α-H-α-amino acid amide racemase was partially purified from the *E. coli* DH10B / pOa(1)PLV49B10 CFE in an overall yield of 19%. SDS-PAGE using the NuPAGE system (Invitrogen) under reducing conditions with MES buffer showed that about 50% of the protein in the preparation after gel filtration consisted of the racemase.

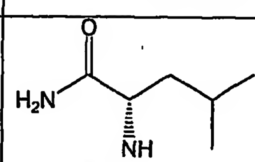
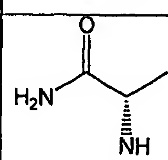
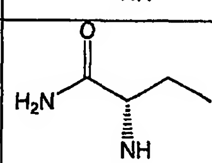
Substrate specificity determination

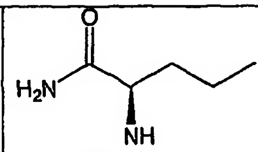
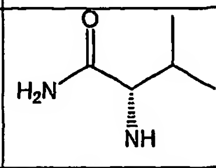
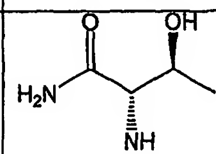
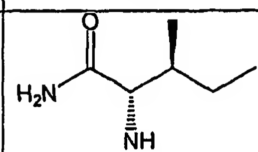
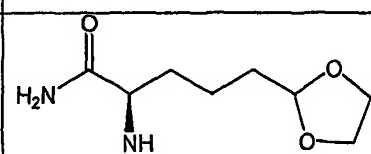
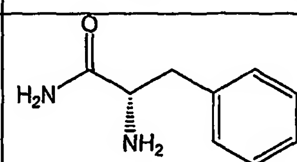
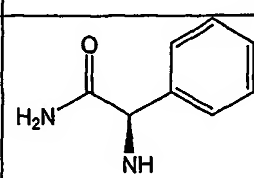
The substrate specificity of the *O. anthropi* IA α-H-α-amino acid

amide racemase was determined using the partially purified enzyme preparation obtained by the protocol described above. Reaction mixtures of 1 ml total volume were prepared by mixing 772 µl of 66.6 mM HEPES-NAOH, pH 8.0, 10 µl of 0.1 M dithiothreitol, 18 µl of a solution containing 20 mg/ml BSA and 1 mM pyridoxal-5-phosphate, and 100 µl of a 0.75 M substrate solution, pH 8.0. After equilibration for 5 minutes at 37°C, the reactions were started by the addition of 100 µl of the enzyme solution. Reaction mixtures were incubated at 37°C without shaking. After regular time intervals, samples of 50 µl were taken and transferred into vials containing 950 µl of 1 M H_3PO_4 to stop the reaction. After sufficient dilution of the samples in 0.4 M borate buffer pH 9.4, the mixtures obtained were analyzed using HPLC to determine the exact concentrations of two amino acid amide enantiomers according to the method of example 7.

Initial activity for the different substrates tested was calculated from the linear parts of the progress curves, in which the amount of the amino acid amide enantiomer formed (so with a stereochemistry opposite to the substrate molecule) was plotted against the reaction time. The results of this experiment are given in table 7.

Table 7. Relative initial activity of the partially purified α-H-α-amino acid amide racemase from *O. anthropi* IA towards different enantiomerically pure α-H-α-amino acid amides.

Substrate	Structure	Rel. initial activity (%)[a]
L-leucine amide		100
L-alanine amide		350
L-2-aminobutyric acid amide		641

D-norvaline amide		341
L-valine amide		4.5
LL-threonine amide		100
LL-isoleucine amide		4.5
D-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide		9.1
L-phenylalanine amide		4.5
D-phenylglycine amide		9.1

[a] All initial activities are given relative to the activity towards L-leucine amide

The data in table 7 clearly show that the α-H-α-amino acid amide racemase from *O. anthropi* IA has a relaxed substrate specificity, because activity was found towards amino acid amides with a short and long chain alkyl side chain, that can optionally be substituted at its C_β atom, as well as towards amino acid amides with an aryl side chain.

Example 9

Determination of the substrate specificity of the α-H-α-amino acid amide racemase from *Arthrobacter nicotianae* NCIMB 41126 using cell free extract from *E. coli* DH10B / pAn(1)PLV36D06

Preparation of cell free extract

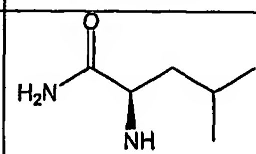
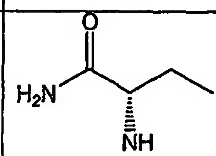
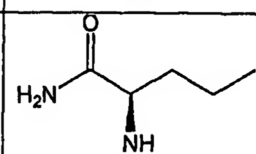
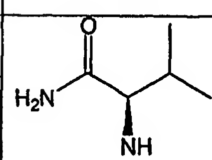
Preparation of the CFE of *E. coli* DH10B / pAn(1)PLV36D06 was performed as described in example 7 for *E. coli* TOP10 / pKEC_AZAR_3.

Substrate specificity determination

Before applying the CFE from *E. coli* DH10B / pAn(1)PLV36D06 in the reactions with different enantiomerically pure α-H-α-amino acid amides to determine the substrate specificity of the *A. nicotianae* α-H-α-amino acid amide racemase, the *E. coli* amidases / aminopeptidases in this CFE were inhibited by a pre-treatment with an inhibitor cocktail. One tablet of Complete Mini Protease Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany) was dissolved in 9 ml of the CFE followed by an incubation of 1 hour at room temperature. Next, reaction mixtures of 10 ml each (including the CFE) were prepared containing 50 mM HEPES-NaOH, pH 8.0, 20 µM pyridoxal-5-phosphate, 0.4 mg/ml BSA, 1 mM dithiothreitol and 75 mM of one of the amino acid amides tested. Reactions were started by the addition of 1 ml of the pre-treated CFE of *E. coli* DH10B / pAn(1)PLV36D06 or *E. coli* TOP10 / pBAD/*Myc*-HisC (blank) and were incubated at 37°C. At regular time intervals samples of 1 ml were taken and transferred to vials containing 1 ml of 1M H_3PO_4 to stop the reaction. After sufficient dilution of the samples in 0.4 M borate buffer pH 9.4, the mixtures obtained were analyzed using HPLC to determine the exact concentrations of the two amino acid and two amino acid amide enantiomers according to the method of example 7. Also the calculation of the initial activity of the CFE towards the different substrate α-H-α-amino acid amides was done as described in example 7, including the correction for potential hydrolysis of the amide substrate and/or product by amidases / aminopeptidases that were not completely inhibited by the pre-incubation of the CFE with the inhibitor cocktail.

As already found in example 7, also the pretreated CFE of *E. coli* TOP10 / pBAD/*Myc*-HisC did not show any racemization of the enantiopure α-H-α-amino acid amide substrates used. The results of this experiment with the CFE from *E. coli* TOP10 / pAn(1)PLV36D06 are given in table 8.

Table 8. Relative initial activity of CFE from *E. coli* DH10B / pAn(1)PLV36D06 towards different enantiomerically pure α-H-α-amino acid amides

Substrate	Structure	Rel. initial activity (%)[a]
D-leucine amide		100
L-2-aminobutyric acid amide		76
D-norvaline amide		107
D-valine amide		45

[a] All initial activities are given relative to the activity towards D-leucine amide

The results presented in table 8 clearly prove that the *A. nicotianae* NCIMB 41126 α-H-α-amino acid amide racemase has not only activity towards leucine amide, but also towards other α-H-α-amino acid amides.

<u>Example 10</u>

<u>α-H-α-amino acid amide racemase activity test using DL-2-aminobutyric acid amide as substrate and CFE from *E. coli* DH10B / pOa(1)PLV49B10 containing the α-H-α-amino acid amide racemase from *O. anthropi* IA</u>

Preparation of the cell free extract

Cultivation of *E. coli* DH10B / pOa(1)PLV49B10 as well as the preparation of the cell free extract were performed in a manner comparable to example 7. Also in this case, *E. coli* TOP10 / pBAD/*Myc*-HisC was treated in exactly the same way to serve as negative control.

α-H-α-Amino acid amide racemase activity towards DL-2-aminobutyric acid amide

To determine the activity of the CFE from *E. coli* DH10B / pOa(1)PLV49B10 and *E. coli* TOP10 / pBAD/*Myc*-HisC, reaction mixtures of 10 ml each were prepared containing 50 mM HEPES-NaOH, pH 8.0, 20 µM pyridoxal-5-phosphate, 0,4 mg/ml BSA, 1 mM dithiothreitol, and 75 mM of DL-2-aminobutyric acid amide. Reactions were started by the addition of 1 ml of the thawed CFE of *E. coli* DH10B / pOa(1)PLV49B10 or *E. coli* TOP10 / pBAD/*Myc*-HisC (blank) and were incubated at 37°C for 21.5 h. At regular time intervals samples of 1 ml were taken and transferred to vials containing 1 ml of MeOH to stop the reaction. Analysis of these samples to determine the concentrations L- and D-2-aminobutyric acid and L- and D-2-aminobutyric acid amide was done by HPLC according to the following protocol.

column:	Sumichiral OA5000 from Sumika (150 x 4.6 mm I.D., 5µ) + guard column
eluent:	4mM $CuSO_4$ in water
flow:	1 ml/min.
column temp.:	10°C
inj. volume:	6µl
detection:	fluorescence detection after post-column reaction with *o*-phthalaldehyde and 2-mercaptoethanol in 0.4 M borate buffer with addition of EDTA (wavelength ex=338 nm and em>420 nm)

The results of this experiment are presented in table 9.

Table 9. α-H-α-amino acid amide racemase activity towards DL-2-aminobutyric acid (Abu) amide of CFE from *E. coli* DH10B / pOA(1)PLV49B10 and *E. coli* blank (*E. coli* TOP10 / pBAD/*Myc*-HisC).

Sample code	Incubation time (h)	D-Abu amide (mM)	L-Abu amide (mM)	D-Abu (mM)	L-Abu (mM)	Conversion[a] (%)	e.e.$_{L\text{-}Abu}$ (%)
B	0	41.1	37.5	0.0	2.1	2.6	100
B	0.75	43.2	26.1	0.0	14.4	17.2	100
B	2	42.2	7.2	0.0	30.5	38.2	100
B	4.65	38.9	0.1	0.3	39.4	50.1	98.5
B	8.25	42.4	0.1	0.7	40.9	48.7	96.7
B	21.5	40.9	0.1	1.7	38.3	47.4	91.7
A	0	42.3	40.5	0.0	1.4	1.6	100
A	0.75	39.6	29.3	0.0	12.3	15.2	100
A	2	34.7	17.8	0.0	29.1	35.7	100
A	4.65	20.8	11.5	0.1	52.3	61.8	99.6
A	8.25	12.4	8.6	0.5	64.9	75.1	98.5
A	21.5	6.7	5.8	1.4	71.7	83.9	96.3

[a] Conversion to the L-acid has been calculated using the formula:
Conversion = [L-acid / (L-acid + D-acid + L-amide + D-amide)] x 100%

Strain A: *E. coli* DH10B / pOA(1)PLV49B10
Strain B: *E. coli* TOP10 / pBAD/*Myc*-HisC

From the data in table 9 it becomes clear that the maximal conversion obtained with the CFE from the *E. coli* blank cells (*E. coli* TOP10 / pBAD/*Myc*-HisC) doesn't exceed 50%. Because these cells do not contain an α-H-α-amino acid amide racemase activity, only the L-aminobutyric acid amide will be converted, leaving the other substrate enantiomer untouched. So the maximum theoretical yield in this case is 50% only, as is standard for (enzymatic) kinetic resolution reactions. The data from the last two time points of this series show that the L-amidase / aminopeptidase from *E. coli* TOP10 also slowly converts the D-aminobutyric acid amide when the concentration of the L-substrate approaches zero, resulting in L-aminobutyric acid with an enantiomeric excess of 91.7% after 21.5 hours

of reaction.

With the CFE from *E. coli* DH10B / pOA(1)PLV49B10 containing the α-H-α-amino acid amide racemase from *O. anthropi* iA, clearly also the D-aminobutyric acid amide is converted, leading to an overall conversion to L-aminobutyric acid of 85.4% after 21.5 hours. The enantiomeric excess of the obtained L-aminobutyric acid in the reaction mixture is still well over 95% throughout the reacton. This is caused by the higher L-aminobutyric acid amide concentration at the end of the reaction through action of the α-H-α-amino acid amide racemase.

This experiment proves that by combining the α-H-α-amino acid amide racemase from *O. anthropi* IA with an L-selective amidase / aminopeptidase (as present in e.g. the CFE from *E. coli* TOP10 cells) DL-α-H-α-amino acid amides can be converted into L-α-H-α-amino acids in more than 50% yield and excellent enantiomeric excess.

Example 11

α-H-α-Amino acid amide racemase activity test using DL-2-aminobutyric acid amide as substrate and CFE from *E. coli* DH10B / pOa(1)PLV49B10 combined with the L-aminopeptidase from *Pseudomonas putida* ATCC 12633

In this experiment, the *E. coli* DH10B / pOA(1)PLV49B10 CFE from example 11 was used. It was combined with a preparation containing the L-aminopeptidase from *P. putida* ATCC 12633. This is the same L-enantioselective enzyme as was used as L-aminopeptidase help solution in the screening of the *O. anthropi* IA and *A. nicotianae* NCIMB 41126 gene libraries (examples 3 and 3A).

A reaction mixture of 10 ml (total volume including enzyme preparations) was prepared containing 50 mM HEPES-NaOH, pH 8.0, 20 μM pyridoxal-5-phosphate, 0,4 mg/ml BSA, 1 mM dithiothreitol, and 75 mM of DL-2-aminobutyric acid amide. Reaction was started by the addition of 0.4 ml of the thawed CFE of *E. coli* DH10B / pOa(1)PLV49B10 and 0.17 ml of a solution containing L-aminopeptidase from *P. putida* ATCC 12633. The reaction mixture was incubated at 37°C for 18 h. At certain time intervals samples of 1 ml were taken and transferred to vials containing 1 ml of MeOH to stop the reaction. Analysis of these samples to determine the concentrations L- and D-2-aminobutyric acid and L- and D-2-aminobutyric acid amide was done by HPLC according to the protocol of example 11. The results of this experiment are presented in table 10.

Table 10. α-H-α-amino acid amide racemase activity towards DL-2-aminobutyric acid (Abu) amide of CFE from *E. coli* DH10B / pOA(1)PLV49B10 combined with the L-aminopeptidase from *Pseudomonas putida* ATCC 12633.

Incubation time (h)	D-Abu amide (mM)	L-Abu amide (mM)	D-Abu (mM)	L-Abu (mM)	Conversion (%)	e.e.$_{L\text{-}Abu}$ (%)
0	36.2	34.1	0.0	0.5	0.7	100
0.5	34.6	22.2	0.0	11.7	17.1	100
1.0	36.4	7.3	0.1	32.2	42.4	99.4
1.5	33.0	2.7	0.0	38.5	51.9	100
2.0	30.4	1.7	0.0	41.9	56.7	100
18	0.4	0.0	1.6	70.8	97.3	95.7

The data in table 10 show that by the combination of the α-H-α-amino acid amide racemase from *O. anthropi* IA and the L-aminopeptidase from *P. putida* ATCC 12633 an extremely high conversion to L-2-aminobutyric acid (97.3%) can be obtained. This conversion obtained is very close to the maximal theoretical value that can be obtained for a dynamic kinetic resolution (i.e. 100%). Furthermore, the enantiomeric excess of the L-2-aminobutyric acid is still above 95%.

Example 12

α-H-α-Amino acid amide racemase activity test using DL-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide as substrate and CFE from *E. coli* DH10B / pOa(1)PLV49B10 combined with the L-aminopeptidase from *Pseudomonas putida* ATCC 12633

In a very similar set-up, the combined activity of the α-H-α-amino acid amide racemase from *O. anthropi* IA and the L-aminopeptidase from *P. putida* ATCC 12633 was determined towards the more complex substrate DL-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide. The molecular structure of this amide can be found in the substrate table in example 8. In this experiment 1 ml of the thawed CFE from *E. coli* DH10B / pOa(1)PLV49B10 was used together with 1 ml of a solution containing L-aminopeptidase from *P. putida* ATCC 12633. Reaction proceeded for 120 hours. Analysis of these samples to determine the concentrations L- and D-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid and L- and D-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide was done by HPLC according to the following protocol.

column: Crownether Cr(-) (150 mm x 4.0 mm ID)
eluent: Perchloric acid in water, pH = 1.0
flow: 1.2 ml/min.
column temp.: 18°C
inj. volume: 20 µl
detection: fluorescence detection after post-column reaction with o-phthalaldehyde and 2-mercaptoethanol in 0.4 M borate buffer. (wavelength ex=338 nm and em>420 nm)

The results of this experiment are presented in table 11.

Table 11. α-H-α-amino acid amide racemase activity towards DL-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide of CFE from *E. coli* DH10B / pOA(1)PLV49B10 combined with the L-aminopeptidase from *Pseudomonas putida* ATCC 12633.

Incubation time (h)	D-Abu amide (mM)	L-Abu amide (mM)	D-Abu (mM)	L-Abu (mM)	Conversion (%)	e.e.$_{L\text{-}Abu}$ (%)
0	36.6	36.5	0.0	4.3	5.6	100
3.6	35.7	2.7	0.0	37.6	49.5	100
22.1	29.9	1.0	0.1	40.3	56.6	99.5
53.2	27.4	0.1	0.1	43.5	61.1	99.5
120.2	23.7	0.1	0.4	46.0	65.5	98.2

The data in table 11 show that by the combination of the α-H-α-amino acid amide racemase from O. *anthropi* IA and the L-aminopeptidase from *P. putida* ATCC 12633 over 65% conversion of the DL-2-amino-5-[1,3]dioxolan-2-yl-pentanoic acid amide to the L-acid is be obtained in 120 hours of reaction. The enantiomeric excess of the L-acid formed is higher than 98%.

The results obtained in this experiment prove that a dynamic kinetic resolution by combining the α-H-α-amino acid amide racemase from O. *anthropi* IA with an L-selective amidase / aminopeptidase (as e.g. the L-aminopeptidase from *P. putida* ATCC 12633) is also possible for more complex α-H-α-amino acid amides containing functionilized side chains.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

DSM Fine Chemicals – Advanced Synthesis and Catalysis (DFC-ASC)
DSM
P O Box 18
6160 MD Geleen
The Netherlands

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Arthrobacter nicotianae	NCIMB 41126

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

- [ ] a scientific description
- [x] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 8 May 2002 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.,

Address: 23 St Machar Drive
Aberdeen
AB24 3RY
Scotland.

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):

Terence Dando

Date: 24 May 2002

[1] Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

DSM Fine Chemicals – Advanced Synthesis and Catalysis (DFC-ASC) DSM P O Box 18 6160 MD Geleen The Netherlands	**INTERNATIONAL FORM** **RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT** **issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page**

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Agrobacterium rhizogenes Na	NCIMB 41127

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☐ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

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Address:23 St Machar Drive Aberdeen AB24 3RY Scotland.	Terence Dando Date:24 May 2002

[1] Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

DSM Fine Chemicals – Advanced Synthesis and Catalysis (DFC-ASC)
DSM
P O Box 18
6160 MD Geleen
The Netherlands

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
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I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Agrobacterium rhizogenes Bi	NCIMB 41128

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

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[X] a proposed taxonomic designation

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INTERNATIONAL FORM

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issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Ochrobactrum anthropi IA	NCIMB 41129

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

- [ ] a scientific description
- [X] a proposed taxonomic designation

(Mark with a cross where applicable)

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AB24 3RY
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Terence Dando

Date: 24 May 2002

[1] Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

CLAIMS

1. Isolated polypeptide having α-H-α-amino acid amide racemase activity and having a degree of identity with the amino acid sequence presented in SEQ ID: NO. 2 of at least about 35%, preferably of at least about 40%, more preferably of at least about 50%, even more preferably of at least about 55%, in particular of at least about 65%, more in particular of at least about 75%, even more in particular of at least about 85%, even more in particular of at least about 90%, even more in particular of at least about 95%, most in particular of at least about 97%.
2. Isolated polypeptide having α-H-α-amino acid amide racemase activity and having a degree of identity with the amino acid sequence presented in SEQ ID: NO. 9 of at least about 50%, preferably of at least about 60%, more preferably of at least about 70%, even more preferably of at least about 80%, in particular of at least about 90%, more in particular of at least about 95%, most in particular of at least about 97%.
3. Isolated polypeptides having α-H-α-amino acid amide racemase activity, which are encoded by nucleic acid sequences which hybridize under low stringency conditions, preferably under medium stringency conditions, more preferably under high stringency conditions, most preferably under very high stringency conditions with SEQ ID: NO. 1 or a complementary strand thereof or with SEQ ID: NO. 8 or a complementary strand thereof.
4. Isolated polypeptides having α-H-α-amino acid amide racemase activity which display immunological cross reactivity with an antibody raised against a fragment of the amino acid sequence according to SEQ ID: NO. 2 or SEQ ID: NO. 9.
5. Isolated polypeptides or fragments thereof of at least 100 amino acids, preferably 125-350 amino acids, more preferably 200-300 amino acids, with α-H-α-amino acid amide racemase activity
6. Isolated fusion protein made by expression of a nucleic acid sequence encoding a polypeptide according to any of claims 1-5 operatively linked to one or more nucleic acid sequences, which encode (a) marker polypeptide(s).
7. Nucleic acid sequence encoding a polypeptide according to any of claims 1-5 or a fusion protein according to claim 6.

8. Vector comprising a nucleic acid sequence according to claim 7.

9. Host cell comprising and expressing a nucleic acid sequence according to claim 7 or a vector according to claim 8.

10. Method for isolating a microorganism displaying α-H-α-amino acid amide racemase activity, comprising the steps of:
    a) Culturing, in one or more transfer steps, a microorganism containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source,
    b) Testing the thus obtained microorganism(s) for α-H-α-amino acid amide racemase activity.

11. Microorganism obtainable by the method of claim 11.

12. *Agrobacterium rhizogenes* Na deposited under number NCIMB 41127, *Agrobacterium rhizogenes* Bi deposited under number NCIMB 41128, *Arthrobacter nicotianae* deposited under number NCIMB 41126, *Ochrobactrum anthropi* IA deposited under number NCIMB 41129.

13. Method for isolating a nucleic acid sequence encoding a polypeptide with α-H-α-amino acid amide racemase activity, comprising the steps of:
    a) Culturing, in one or more transfer steps, a microorganism containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source,
    b) Testing the thus obtained microorganism(s) for α-H-α-amino acid amide racemase activity.
    c) Isolating the nucleic acid sequence from the obtained microorganism(s) in a manner known per se.

14. Nucleic acid sequence obtainable by the method of claim 13.

15. Method for the production of a polypeptide with α-H-α-amino acid amide racemase activity, comprising the steps of:
    a) Culturing, in one or more transfer steps, a microorganism containing sample in or on a suitable growth medium containing D-α-amino-ε-caprolactam or a mixture of D- and L-α-amino-ε-caprolactam as a or as the sole nitrogen source,
    b) Testing the thus obtained microorganism(s) for α-H-α-amino acid amide

racemase activity.

c) Isolating the nucleic acid sequence from the obtained microorganism(s) in a manner known per se.

d) Expressing the nucleic acid sequence in a suitable host to produce a polypeptide with α-H-α-amino acid amide racemase activity.

16. Polypeptide with α-H-α-amino acid amide racemase activity obtainable by the method of claim 15.

17. Process for the racemization of an enantiomerically enriched α-H-α-amino acid amide, wherein the racemization is performed in the presence of a polypeptide according to any of claim 1-6 or 16, in the presence of a host cell according to claim 9 or in the presence of a microorganism according to claim 11 or claim 12.

18. Process for the preparation of an enantiomerically enriched α-H-α-amino acid from a mixture of the corresponding D- and L-α-H-α-amino acid amides or for the preparation of an L-α-H-α-amino acid from the corresponding D-α-H-α-amino acid amide or for the preparation of an D-α-H-α-amino acid from the corresponding L-α-H-α-amino acid amide, wherein the process is performed in the presence of an enantioselective amidase and in the presence of a polypeptide according to any of claim 1-6 or 16 or in the presence of a host cell according to claim 9 or in the presence of a microorganism according to claim 11 or claim 12.

SEQUENCE LISTING

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<120> POLYPEPTIDES HAVING alpha-H-alpha-AMINO ACID AMIDE RACEMASE ACTIVITY AND NUCLEIC ACIDS ENCODING THE SAME

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<213> Arthrobacter nicotianae NCIMB 41126

<400> 3
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cgaacgggtg agtaacacgt gagtaacctg cccccgactc tgggataagc ccgggaaact 120

gggtctaata ccggatattc acttccttcc gcatggaggg tggtggaaag gtcttttccg 180

gtcggggatg gactcgcggc ctatcagctt gttggtgggg tagtggccca ccaaggcgac 240

gacgggtagc cggcctgaga gggtgaccgg ccacactggg actgagacac ggcccagact 300

cctacgggag gcagcagtgg ggaatattgc acaatgggcg caagcctgat gcagcgacgc 360

cgcgtgaggg atgacggcct tcgggttgta aacctctttc agtagggaag aagcgaaagt 420

gacggtacct gcagaa 436

<210> 4
<211> 400
<212> DNA
<213> Agrobacterium rhizogenes Bi NCIMB 41128

<400> 4
ggcggcaggc ttaacacatg caagtcgagc gccccgcaag gggagcggca gacgggtgag 60

taacgcgtgg gaatctaccc ttttctacgg aataacgcag ggaaacttgt gctaataccg 120

tatacgccct tcgggggaaa gatttatcgg gaaaggatga gcccgcgttg gattagctag 180

ttggtggggt aaaggcctac caaggcgacg atccatagct ggtctgagag gatgatcagc 240

cacattggga ctgagacacg gcccaaactc ctacgggagg cagcagtggg gaatattgga 300

caatgggcgc aagcctgatc cagccatgcc gcgtgagtga tgaaggccct agggttgtaa 360

agctctttca ccggtgaaga taatgacggt aaccggagaa 400

<210> 5
<211> 381
<212> DNA
<213> Agrobacterium rhizogenes Na NCIMB 41127

<400> 5
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gcccttcggg ggaaagattt atcgggaaag gatgagcccg cgttggatta gctagttggt 180

ggggtaaagg cctaccaagg cgacgatcca tagctggtct gagaggatga tcagccacat 240

tgggactgag acacggccca aactcctacg ggaggcagca gtggggaata ttggacaatg 300

ggcgcaagcc tgatccagcc atgccgcgtg agtgatgaag gccctagggt tgtaaagctc 360

tttcaccggt gaagataatg a 381

<210> 6
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic DNA

<400> 6
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<210>  7
<211>  39
<212>  DNA
<213>  Artificial Sequence

<220>
<223>  Synthetic DNA

<400>  7
gcctcacccg ggttaccaca tcataaaatg ggcgacatc                              39


<210>  8
<211>  1454
<212>  DNA
<213>  Arthrobacter nicotianae NCIMB 41126

<220>
<221>  CDS
<222>  (80)..(1417)
<223>  Amino Acid Amide Racemase encoding sequence


<400>  8
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ggagcgccga ggagggcac atg ctt gaa gac tct ctc tac gcc cgc gac ggg      112
                    Met Leu Glu Asp Ser Leu Tyr Ala Arg Asp Gly
                    1               5                   10

cgc gtt atc gca ggc gta gag aag ctg cgc ttc ttt ccg ctg gag act      160
Arg Val Ile Ala Gly Val Glu Lys Leu Arg Phe Phe Pro Leu Glu Thr
            15                  20                  25

gcc tcc ggc cgg ggc agc atg ctc gtc gag cca ggt ggc agg gaa ttg      208
Ala Ser Gly Arg Gly Ser Met Leu Val Glu Pro Gly Gly Arg Glu Leu
        30                  35                  40

ttc gac ttc agc gcc agc tgg acg gct gca ggg ctg ggg cac ggg aac      256
Phe Asp Phe Ser Ala Ser Trp Thr Ala Ala Gly Leu Gly His Gly Asn
    45                  50                  55

cct gaa atc acc gcg gcc att gca cga gcc gct gtt gat tct ccc ggc      304
Pro Glu Ile Thr Ala Ala Ile Ala Arg Ala Ala Val Asp Ser Pro Gly
60                  65                  70                  75

gca tct atc ctg tcg gca aca cac tcg gaa gct gtc gga ctg gcc gaa      352
Ala Ser Ile Leu Ser Ala Thr His Ser Glu Ala Val Gly Leu Ala Glu
                80                  85                  90

cga ctc ctg gac atg gtt ccg acg cga agg tcc ggc ccc ggt ggg cgg      400
Arg Leu Leu Asp Met Val Pro Thr Arg Arg Ser Gly Pro Gly Gly Arg
            95                  100                 105

cgt gtc tat ctt ggc cac gcc gga acc gac tcg aat gat gta gct atc      448
Arg Val Tyr Leu Gly His Ala Gly Thr Asp Ser Asn Asp Val Ala Ile
        110                 115                 120
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aga ggc tgc cgc cat gct tcc gga agg cca gga gtc atc gcc ttc gaa      496
Arg Gly Cys Arg His Ala Ser Gly Arg Pro Gly Val Ile Ala Phe Glu
    125                 130                 135

ggc ggg tat cat ggc ggg ctc ggc atc gcc cag cgc ata tcg ggt gtc      544
Gly Gly Tyr His Gly Gly Leu Gly Ile Ala Gln Arg Ile Ser Gly Val
140                 145                 150                 155

cat gtg gat tcc ggc gtt cct gcc gac ccc cat gta gcg ttc gtc ccc      592
His Val Asp Ser Gly Val Pro Ala Asp Pro His Val Ala Phe Val Pro
                160                 165                 170

tat cct gat ctg ttc cgc ccg cac acg ggt gac ccg gag aca gtt ctg      640
Tyr Pro Asp Leu Phe Arg Pro His Thr Gly Asp Pro Glu Thr Val Leu
            175                 180                 185

ccg gac gtg ctg acg cgc gtc cgg cag aac ctc caa cgg ggc atg acc      688
Pro Asp Val Leu Thr Arg Val Arg Gln Asn Leu Gln Arg Gly Met Thr
        190                 195                 200

gca gcc gtc atc gtc gaa cct ctc ctc tcc gac ggc ggc gtc atc gtg      736
Ala Ala Val Ile Val Glu Pro Leu Leu Ser Asp Gly Gly Val Ile Val
    205                 210                 215

ccg ccc ccg gag ttc ctc cgt ggt cta agg gaa ctg tgc gac gcg cac      784
Pro Pro Pro Glu Phe Leu Arg Gly Leu Arg Glu Leu Cys Asp Ala His
220                 225                 230                 235

aac gca tac ctc atc gtg gac gag gtc aaa gta ggg ctc ggg cgc act      832
Asn Ala Tyr Leu Ile Val Asp Glu Val Lys Val Gly Leu Gly Arg Thr
                240                 245                 250

ggc agc ctt cac gcc ttc gaa cat gac ggc atc ctg ccg gac att gtc      880
Gly Ser Leu His Ala Phe Glu His Asp Gly Ile Leu Pro Asp Ile Val
            255                 260                 265

acg ctc gga aaa gtc ctt ggt ggc ggg ctc ccc ctt tcc gcg gcc atc      928
Thr Leu Gly Lys Val Leu Gly Gly Gly Leu Pro Leu Ser Ala Ala Ile
        270                 275                 280

ggt ccg tcg gaa gtt ctc gac cgg ccg gtt gcc tcg gcg tta atg acg      976
Gly Pro Ser Glu Val Leu Asp Arg Pro Val Ala Ser Ala Leu Met Thr
    285                 290                 295

act aca ggc aat ccc atc tcc tgc gcg gct ggc cgt gcc gca gtc gaa     1024
Thr Thr Gly Asn Pro Ile Ser Cys Ala Ala Gly Arg Ala Ala Val Glu
300                 305                 310                 315

ata gtg tgc aga ggc gac gtc atc cgg aat gcg gcc gag cgc ggc gag     1072
Ile Val Cys Arg Gly Asp Val Ile Arg Asn Ala Ala Glu Arg Gly Glu
                320                 325                 330

caa atc aga gac ctg ctc gcc gcc tat gca aga gaa acc ggg cgg cct     1120
Gln Ile Arg Asp Leu Leu Ala Ala Tyr Ala Arg Glu Thr Gly Arg Pro
            335                 340                 345
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ggc gca gcc cac gtt ggt gat gtc cgc ggc cgc ggc ctc tcc atc ggc      1168
Gly Ala Ala His Val Gly Asp Val Arg Gly Arg Gly Leu Ser Ile Gly
        350                 355                 360

atc gag att gtc acg gac cgg gac gaa aat gtc agc gat ccg ggg ctc      1216
Ile Glu Ile Val Thr Asp Arg Asp Glu Asn Val Ser Asp Pro Gly Leu
    365                 370                 375

acc gcc aaa gcc gtc tac cgt gcc tgg gag ctt ggc gtg gtc gtg cat      1264
Thr Ala Lys Ala Val Tyr Arg Ala Trp Glu Leu Gly Val Val Val His
380                 385                 390                 395

cca gtg cgc ggc aat gtc ctt gaa ctc aca ccg ccg ctc aca gtg tca      1312
Pro Val Arg Gly Asn Val Leu Glu Leu Thr Pro Pro Leu Thr Val Ser
                400                 405                 410

gca gac gag gtg cag cag gcc atg gac ctc ctg acc tgc gcg cta gat      1360
Ala Asp Glu Val Gln Gln Ala Met Asp Leu Leu Thr Cys Ala Leu Asp
            415                 420                 425

gac gcc gct cgg ggc ctc gtg agc gat gag cag atc gct ccc tat gct      1408
Asp Ala Ala Arg Gly Leu Val Ser Asp Glu Gln Ile Ala Pro Tyr Ala
        430                 435                 440

ggt tgg tga cgtgaaaaaa ccgccggaga gcggaaccgg cgtgagc                 1454
Gly Trp
    445


<210>  9
<211>  445
<212>  PRT
<213>  Arthrobacter nicotianae NCIMB 41126

<400>  9

Met Leu Glu Asp Ser Leu Tyr Ala Arg Asp Gly Arg Val Ile Ala Gly
1               5                   10                  15


Val Glu Lys Leu Arg Phe Phe Pro Leu Glu Thr Ala Ser Gly Arg Gly
            20                  25                  30


Ser Met Leu Val Glu Pro Gly Gly Arg Glu Leu Phe Asp Phe Ser Ala
        35                  40                  45


Ser Trp Thr Ala Ala Gly Leu Gly His Gly Asn Pro Glu Ile Thr Ala
    50                  55                  60


Ala Ile Ala Arg Ala Ala Val Asp Ser Pro Gly Ala Ser Ile Leu Ser
65                  70                  75                  80


Ala Thr His Ser Glu Ala Val Gly Leu Ala Glu Arg Leu Leu Asp Met
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                85                  90                  95

Val Pro Thr Arg Arg Ser Gly Pro Gly Gly Arg Arg Val Tyr Leu Gly
            100                 105                 110

His Ala Gly Thr Asp Ser Asn Asp Val Ala Ile Arg Gly Cys Arg His
        115                 120                 125

Ala Ser Gly Arg Pro Gly Val Ile Ala Phe Glu Gly Gly Tyr His Gly
    130                 135                 140

Gly Leu Gly Ile Ala Gln Arg Ile Ser Gly Val His Val Asp Ser Gly
145                 150                 155                 160

Val Pro Ala Asp Pro His Val Ala Phe Val Pro Tyr Pro Asp Leu Phe
                165                 170                 175

Arg Pro His Thr Gly Asp Pro Glu Thr Val Leu Pro Asp Val Leu Thr
            180                 185                 190

Arg Val Arg Gln Asn Leu Gln Arg Gly Met Thr Ala Ala Val Ile Val
        195                 200                 205

Glu Pro Leu Leu Ser Asp Gly Gly Val Ile Val Pro Pro Pro Glu Phe
    210                 215                 220

Leu Arg Gly Leu Arg Glu Leu Cys Asp Ala His Asn Ala Tyr Leu Ile
225                 230                 235                 240

Val Asp Glu Val Lys Val Gly Leu Gly Arg Thr Gly Ser Leu His Ala
                245                 250                 255

Phe Glu His Asp Gly Ile Leu Pro Asp Ile Val Thr Leu Gly Lys Val
            260                 265                 270

Leu Gly Gly Gly Leu Pro Leu Ser Ala Ala Ile Gly Pro Ser Glu Val
        275                 280                 285

Leu Asp Arg Pro Val Ala Ser Ala Leu Met Thr Thr Thr Gly Asn Pro
    290                 295                 300

Ile Ser Cys Ala Ala Gly Arg Ala Ala Val Glu Ile Val Cys Arg Gly
305                 310                 315                 320
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Asp Val Ile Arg Asn Ala Ala Glu Arg Gly Glu Gln Ile Arg Asp Leu
325 330 335

Leu Ala Ala Tyr Ala Arg Glu Thr Gly Arg Pro Gly Ala Ala His Val
340 345 350

Gly Asp Val Arg Gly Arg Gly Leu Ser Ile Gly Ile Glu Ile Val Thr
355 360 365

Asp Arg Asp Glu Asn Val Ser Asp Pro Gly Leu Thr Ala Lys Ala Val
370 375 380

Tyr Arg Ala Trp Glu Leu Gly Val Val Val His Pro Val Arg Gly Asn
385 390 395 400

Val Leu Glu Leu Thr Pro Pro Leu Thr Val Ser Ala Asp Glu Val Gln
405 410 415

Gln Ala Met Asp Leu Leu Thr Cys Ala Leu Asp Asp Ala Ala Arg Gly
420 425 430

Leu Val Ser Asp Glu Gln Ile Ala Pro Tyr Ala Gly Trp
435 440 445

<210> 10
<211> 67
<212> DNA
<213> Artificial Sequence

<220>
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<400> 10
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cggggag 67

<210> 11
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
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<400> 11

ggggaccact ttgtacaaga aagctgggtt caccaaccag catagggagc gatctg 56

<210> 12
<211> 100
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic DNA

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gtttttttggg ctaacacaag tttgtacaaa aaagctgaac 100

<210> 13
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Synthetic DNA

<400> 13
ttgttctacg taaccacttt gtacaagaaa gctgaac 37

INTERNATIONAL SEARCH REPORT

PCT/NL 03/00423

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P41/00 C12N9/90 C12N15/62 C12N1/20 C12N1/21
C12Q1/04 C12P13/04 //(C12P13/04,C12R1:025)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN DEN TWEEL W J J ET AL: "Ochrobactrum anthropi NCIMB 40321: A new biocatalyst with broad-spectrum L-specific amidase activity." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 39, no. 3, 1993, pages 296-300, XP002212107 ISSN: 0175-7598 the whole document ---	10,11
X	FUKUMURA T: "BACTERIAL RACEMIZATION OF ALPHA AMINO-EPSILON CAPROLACTAM" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 41, no. 8, 1977, pages 1321-1326, XP001074251 EN ISSN: 0002-1369 the whole document ---	10,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2003	25/08/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Aslund, J

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 494 716 A (DSM NV) 15 July 1992 (1992-07-15) the whole document ---	11
X	US 5 100 782 A (KLAGES UWE ET AL) 31 March 1992 (1992-03-31) cited in the application the whole document ---	3
X	EP 0 383 403 A (STAMICARBON ;NOVONORDISK AS (DK)) 22 August 1990 (1990-08-22) cited in the application the whole document ---	3
A	KAMPHUIS ET AL.: "The Production and Uses of Optically Pure Natural and Unnatural Amino Acids." CHIRALITY IN INDUSTRY. EDITED BY COLLINS, SHELDRAKE AND CROSBY. JOHN WILEY & SONS LTD, 1992, pages 187-208, XP008006821 page 201 -page 206 ---	
A	WO 86 07386 A (NOVO INDUSTRI AS) 18 December 1986 (1986-12-18) ---	
A	KAPTEIN B ET AL: "Enantiopure Clpha-tetrasubstituted alpha-amino acids. Chemo-enzymatic synthesis and application to turn-forming peptides" TETRAHEDRON, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 57, no. 30, 23 July 2001 (2001-07-23), pages 6567-6577, XP004275079 ISSN: 0040-4020 ---	
A	KATO Y ET AL: "FIRST STEREOSELECTIVE SYNTHESIS OF D AMINO ACID N-ALKYL AMIDE CATALYZED BY D AMINOPEPTIDASE" TETRAHEDRON, vol. 45, no. 18, 1989, pages 5743-5454, XP001074248 ISSN: 0040-4020 ---	
A	TORNOE C W ET AL: "Enzymatic and chiral HPLC resolution of alpha-azido acids and amides" TETRAHEDRON: ASYMMETRY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 11, no. 5, 24 March 1999 (1999-03-24), pages 1239-1248, XP004195821 ISSN: 0957-4166 the whole document --- -/--	9

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AHMED S A ET AL: "PROPERTIES OF ALPHA AMINO-EPSILON CAPROLACTAM RACEMASE EC-5.1.1.- FROM ACHROMOBACTER-OBAE" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol. 47, no. 8, 1983, pages 1887-1894, XP001074250 ISSN: 0002-1369 cited in the application -----	

INTERNATIONAL SEARCH REPORT

PCT/NL 03/00423

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11 (partially)
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 11 (partially)

Present claim 11 relates to a product (a microorganism) defined by reference to a desirable characteristic or property, namely the existence of microorganisms that can be isolated by the method of claim 10. The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the microorganism Ochrobactrum, Agrobacterium and Athrobacter as outlined in examples 2 and 3.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

PCT/NL 03/00423

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0494716 A	15-07-1992	NL 9100038 A	03-08-1992
		AT 141952 T	15-09-1996
		CA 2059075 A1	12-07-1992
		DE 69213033 D1	02-10-1996
		DE 69213033 T2	03-04-1997
		DK 494716 T3	10-02-1997
		EP 0494716 A2	15-07-1992
		ES 2093764 T3	01-01-1997
		JP 3135653 B2	19-02-2001
		JP 5030992 A	09-02-1993
		US 5248608 A	28-09-1993
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		AT 119202 T	15-03-1995
		WO 8910969 A1	16-11-1989
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		AU 5965286 A	07-01-1987
		WO 8607386 A1	18-12-1986
		EP 0228392 A1	15-07-1987
		JP 63500004 T	07-01-1988